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# THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

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THE

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# OBITUARY NOTICES

## HAROLD ACKROYD

HAROLD ACKROYD was the son of Mr Edward Ackroyd, for many years chairman of the Southport and Cheshire Lines Extension Railway. His schools were Mintholme College, Southport, and Shrewsbury. He joined Gonville and Caius College, Cambridge, and took his B.A. in 1899. In the following year he attended the lectures and practical classes as then arranged for physiology in Part II of the Natural Sciences Tripos, though, from the first, he had no intention of entering for the examination. In the work of the chemical section I remember how good for his teacher was the critical attitude he displayed towards the methods used in the practical classes. His were criticisms not verbally expressed but set down in his note book in the form of figures. While others were for the most part content to employ a method "as directed" Ackroyd was apt to test first the limits of its accuracy. Then, as later, and always, he was impatient of compromise, and had no use for the second best, in laboratory methods or anything else; but once at work he spared himself no pains to reach the highest grade of accuracy possible under the circumstances.

He went to Guy's Hospital for his clinical training and took his medical degrees in due course. Later he was House-Surgeon at Queen's Hospital, Birmingham, and then worked for a time at the David Lewis Northern Hospital, Liverpool. He possessed ample private means and never attempted ordinary medical practice.

He hesitated a good deal before deciding to devote himself entirely to scientific research though so well qualified to do it. He was fond of travel and had a passion for the sea. While undecided about his career he indulged these tastes freely, going for long voyages in slow boats and exploring Europe pretty thoroughly.

After his marriage he decided to settle down to biochemical research at Cambridge. He went therefore to reside at Royston, and attached himself, first to the Department of Pharmacology, and afterwards to the School of Agriculture where he accepted a post in the newly founded Institute for the study of Animal Nutrition. He had previously held for some time a British

Medical Association Scholarship. He concerned himself chiefly with the subject of purine metabolism. In 1910 he published a paper showing that when urates are perfused through the liver of the dog they are, partly at least, converted into allantoin, without any formation of urea. Allantoin itself was shown to undergo no change[1]. In a later paper[2] he showed that in the case of rabbit's liver the conversion of uric acid into allantoin is complete, the latter proving once more to be an end product. He demonstrated[3], after a careful analysis of various foodstuffs, that the small quantity of allantoin found in human urine may wholly arise from the diet, even when it consists of milk and vegetables only. Ackroyd's work on these lines gave strong support therefore to the views of Wiechowski, then under discussion, by showing that allantoin, while the end product of purine metabolism in animals, is probably not produced at all in the human body.

For a long time while at the School of Agriculture he engaged most patiently in a laborious study of the nitrogenous constituents of root-crops. The results unfortunately have never been published. A few months before the war I proposed to him some conjoint work upon possible precursors of purines in the body. He began the work, and a preliminary paper[4] was published in December, 1916—a paper which, unhappily, Ackroyd never saw.

In February, 1915, he was commissioned as temporary lieutenant in the R.A.M.C., being afterwards promoted captain. He became attached to the R. Berks Regiment and went to France in August, 1915. He was slightly wounded on July 31st, 1917, but remaining on duty was killed in action on August 11th. Before his death he received notification that he had been awarded the Military Cross for conspicuous bravery as a battalion doctor, but the crowning honour of the V.C. was a reward for his final sacrifice. It, together with the M.C., were received from the hands of the King by his widow and one of his small sons. The London *Gazette* thus describes the conduct for which he was granted the V.C.:

"Utterly regardless of danger he worked continuously for many hours up and down and in front of the line, tending the wounded and saving the lives of officers and men. In so doing he had to move across the open under heavy machine gun, rifle, and shell fire. He carried a wounded officer to a place of safety under very heavy fire. On another occasion he went some way in front of our advanced line and brought in a wounded man under continuous sniping and machine gun fire. His heroism was the means of saving many lives, and

[1] This *Journal*, 1910, **5**, 217. [2] *Ib.* 442. [3] *Ib.* 400.
[4] This *Journal*, 1916, **10**, 551.

provided a magnificent example of courage, cheerfulness, and determination to the fighting men in whose midst he was carrying out his splendid work. This gallant officer has since been killed in action."

To one who knew him best as a colleague patiently engaged in the details of laboratory work these eloquent words bring a strange sense of contrast. Ackroyd however brought courage to every task and did everything well.

F. G. Hopkins.

## SAMUEL GEOFFREY SCOTT.

S. G. Scott came up to Magdalen College, Oxford, in 1893, and took the Final Honour School of Physiology in 1897. During his undergraduate career he was taught Histology by Dr (now Professor) Gustav Mann, and became his devoted disciple, not only in methods of technique, but especially in his regard for Histology as a subject of Micro-Chemistry rather than of minute anatomy. This early enthusiasm persisted and increased till it became the sole aim of his scientific life and thought.

After completing his medical studies in London, Scott became demonstrator of Pathology at Leeds in 1903, at first under Dr Trevelyan, and subsequently under Prof. A. S. Grünbaum. From the nature of his teaching he became drawn to pathological research, and he published several papers in the *Journal of Pathology*, the *Lancet* and elsewhere, dealing with the microscopic anatomy of diseased organs. These include "A Case of Sub-Acute Atrophy of the Liver" (1905), "Obstruction Atrophy of the Pancreas" (1907), and other papers on Multiple Neurofibromatosis, Infantile Splenic Anaemia, Leukaemia, and Aplastic Anaemia, some of these being produced in conjunction with Dr W. H. M. Telling.

In 1908 Scott returned to Oxford as lecturer and demonstrator in Histology, and here he at once settled down to the work for which he had so rare and exceptional an aptitude. On the technical side of Histology he was a master, and his microscopical preparations were perfect. Where his profession was concerned he would admit of nothing second rate, if it could be avoided, and if an innocent undergraduate consulted him as to the microscope he should invest in, he was shown a programme of stand, objectives and eye-pieces costing over £100. Yet such was Scott's persuasiveness and enthusiasm that quite a number of the better-endowed undergraduates followed his advice implicitly. Scott practically lived in the laboratory, as he did not trouble about such mundane matters as lunch, and very rarely took a holiday. He

was always ready to spend unlimited time in giving advice to pupils and colleagues, and to pour out his ideas and theories on the subject so dear to his heart, viz., the chemistry of staining. Of almost every dye he knew not only its staining properties, but its chemical constitution, and nothing pleased him better than to cover blackboard or paper with formulae indicating the probable linkage whereby the stain attached itself to the object stained, and the chemical transformations it underwent during bleaching, change of reaction, and so on. He was very careful and cautious in committing himself in print, and so had accumulated a number of not quite complete investigations, which now, alas, will never be finished off and see the light. His chief published work on micro-chemistry includes two important papers on Romanowsky Staining for Blood Cells (*Journ. of Path.*, Vol. 16, 1911, and *Folia Haematologica*, Vol. 12, 1911), which he produced in conjunction with two of his pupils, T. O. Thompson and J. L. Hydrick.

On the outbreak of the war Scott took up a temporary appointment as house surgeon at the Radcliffe Infirmary, Oxford, and later, finding it was possible to obtain a substitute for his teaching work, he thought it his duty to volunteer for a commission in the R.A.M.C. In the early summer of 1917 he went to France, and later on to Italy. Here he succumbed in a few days to an attack of pneumonia. His friends and colleagues can never forget his genial presence, his unfailing good temper and his intense devotion to his work. They offer their deepest sympathy to his widow.

H. M. Vernon.

# I. OBSERVATIONS ON THE PHOSPHOTUNGSTATES OF CERTAIN BASES AND AMINO-ACIDS.

By JACK CECIL DRUMMOND.

*From the Biochemical Laboratory of the Cancer Hospital Research Institute, London.*

(*Received July 18th, 1917.*)

## Introductory.

In spite of the extensive use which is made of phosphotungstic acid as a precipitant for organic bases, comparatively few investigators have studied the individual salts of the acid or made any attempt to define the most suitable conditions for their preparation.

The observations recorded in this communication were made during an attempt to separate complex mixtures of bases and amino-acids by a process depending upon the relative solubilities of their phosphotungstates in various solvents.

Unfortunately the study is to a large extent incomplete, and, since there does not appear to be any likelihood of the opportunity arising whereby the work may be completed, it has been decided to publish the few results which have been obtained in the hope that they may either be of some slight practical utility, or that they may suggest more extensive investigations along similar lines.

Phosphotungstic acid was introduced as a precipitant for organic bases by Drechsel, and since that introduction its use for that purpose has become more and more extensive. References to the use of the acid are scattered so widely apart throughout the literature that it is difficult to review them at all completely. With regard to the acid itself, its preparation is described by Drechsel [1887], and also by Winterstein [1898]. The two methods are practically identical in detail and give a yield of from two to three parts of the acid from four parts of sodium tungstate and one part of sodium phosphate. The acid prepared by this method is usually somewhat impure and

it is necessary in such cases to subject it to a process of purification by ether treatment (Winterstein). Reference to the literature shows that a large series of phosphotungstic acids have been described, mainly by Kehrmann and his co-workers [1887, 1888, 1892, etc.]. These acids possess different proportions of $P_2O_5$ and $WO_3$. Apparently the acid prepared by the process referred to above, which is that sold commercially, forms salts of the type

$$R_3, H_3PO_4, 12WO_3, xH_2O.$$

(R being a monovalent base.)

Gulewitsch [1899] prepared and submitted to analysis pure preparations of arginine phosphotungstate and found that his analytical data satisfied the formula $(C_6H_{14}O_2N_4)_3, 2H_3PO_4, 24WO_3, 10H_2O$.

Similarly Barber [1906] in an investigation of the phosphotungstates of the simpler amino-acids gives a similar formula for:

glycine phosphotungstate $(C_2H_5O_2N)_3, H_3PO_4, 12WO_3$,
alanine „ $(C_3H_7O_2N)_3, H_3PO_4, 12WO_3$,
glutaminic acid „ $(C_5H_9O_4N)_3, 2H_3PO_4, 24WO_3$.

In the case of the salts of aspartic acid and asparagine, however, he suggests formulae of a different type:

asparagine phosphotungstate $(C_4H_8O_3N_2)_5, 2H_3PO_4, 22WO_3$,
aspartic acid „ $(C_4H_7O_4N)_4, 2H_3PO_4, 20\text{–}22WO_3$.

Wechsler [1911] confirmed the formula given by Gulewitsch for arginine phosphotungstate and found that a similar one, $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3$, satisfied the analytical data for the histidine salt.

A considerable number of salts of phosphotungstic acid with various bases and amino-acids have been prepared during the course of this inquiry and in every preparation submitted to analysis the figures have agreed with the composition indicated by the formula:

$$R_3, H_3PO_4, 12WO_3, xH_2O. \quad (R = \text{monovalent base.})$$

With regard to the studies that have been made of the salts themselves there is little to review. Generally speaking they have been concerned with determinations and comparisons of the relative precipitability of various substances by the acid. Gulewitsch [1899] made a careful investigation of arginine phosphotungstate, and determined the conditions for its optimum precipitability. He pointed out the advantage of utilising a slight excess of the precipitant and of conducting the operation in a medium of 5 % sulphuric acid.

Barber [1906] investigated the phosphotungstates of several of the simpler amino-acids, and recorded their probable constitution, relative precipitability and their solubilities in water, alcohol and 50 % alcohol.

Levene and Beatty [1906] also studied the precipitability of several simple amino-acids.

Other investigations of a similar nature are most difficult to collate since they are scattered so widely throughout the mass of literature dealing with the individual bases themselves. The few that have been traced will be considered when the particular compound to which they refer is being discussed.

## Experimental.

### 1. *Conditions under which maximum precipitation is obtained.*

From a consideration of first principles it is recognised that many factors will influence the precipitability of a substance. In the present case the most important of these factors are temperature, the concentration of base, and the degree of acidity of the medium in which the reaction is carried out.

The study of arginine phosphotungstate made by Gulewitsch [1899] included an experimental investigation of the conditions necessary for the optimum precipitation of that compound from a 0·02 % solution of arginine nitrate.

The results of his investigation demonstrated the advisability of using a slight excess of the precipitant and of operating in a medium of 5 % sulphuric acid. When the precipitation occurred in 2·5 or 7 % sulphuric acid, or when too great an excess of the phosphotungstic acid was used precipitation was either incomplete or the precipitate tended to redissolve.

The determinations of precipitability recorded in this paper were made by adopting a technique similar to that employed by Gulewitsch.

A series of numbered, stoppered, 50 cc. volumetric flasks was taken and into each was introduced a known volume of a dilute solution of the base under examination. A 20 % solution of sulphuric acid was then run in to each flask in an amount sufficient to render the contents of the flask of the required degree of acidity when made up to 50 cc. Distilled water was then added in an amount just sufficient to permit the further addition of the required amount of a 30 % solution of phosphotungstic acid in order to bring the volume of liquid in each flask up to 50 cc.

The flasks were then stoppered and shaken vigorously for half a minute,

after which they were allowed to stand at room temperature (19°) for 24 hours with occasional agitation.

The contents of the flasks were filtered and determinations of unprecipitated nitrogen made upon aliquot portions of the filtrates.

The following tables indicate the findings in the study of the precipitation of betaïne and histidine from solutions of their salts.

TABLE I.

*Precipitation of histidine from a dilute solution of histidine hydrochloride by phosphotungstic acid under varying conditions. Figures represent mg. nitrogen unprecipitated.* (*Temperature* 19°.)

| Strength of sulphuric acid present | cc. 30 % phosphotungstic acid solution | | | | | | |
|---|---|---|---|---|---|---|---|
| % | 0 | | 5 | 10 | 15 | 20 | 30 |
| 0 | 11·58 | — | — | — | 5·21 | 6·00 | 6·30 |
| 2 | — | — | — | — | 3·99 | — | — |
| 3·5 | — | — | — | — | 3·90 | — | — |
| 5 | — | 7·96 | 5·25 | 3·60 | 3·65 | 3·50 | 4·90 |
| 7 | — | 7·96 | 5·25 | 4·05 | 4·00 | 3·60 | 5·22 |
| 9 | — | — | — | — | 4·37 | — | — |
| 11 | — | — | — | — | 4·57 | — | — |
| 18 | — | — | — | — | 5·20 | — | 6·33 |

TABLE II.

*Precipitation of betaïne from a dilute solution of betaïne hydrochloride by phosphotungstic acid under varying conditions.* (*Temperature* 19°.)

| Strength of sulphuric acid | cc. 30 % phosphotungstic acid solution | | | | | |
|---|---|---|---|---|---|---|
| % | 0 | 5 | 8 | 10 | 20 | 30 |
| 0 | 3·07 | — | — | 2·05 | — | — |
| 3·5 | — | — | — | 1·54 | — | 2·07 |
| 5 | — | — | 1·77 | 1·43 | 1·46 | 1·90 |
| 7 | — | 2·17 | 1·83 | 1·47 | 1·54 | 1·96 |
| 9 | — | — | — | 1·54 | — | — |
| 18 | — | 2·24 | — | 1·89 | — | — |

From a consideration of these figures it will be recognised that the conclusions are similar to those made for arginine by Gulewitsch, in that the most satisfactory precipitation of these two bases occurs when a slight excess of the reagent is used and when the operation is carried out in 5 % sulphuric acid. Gulewitsch recorded the different appearances of the precipitated arginine phosphotungstate obtained under varying conditions, for the beneficial influence of the slight excess of phosphotungstic acid is not as readily observed from the tables as it is from a study of the precipitates

themselves. Under conditions such as are indicated above the precipitates tend to become more distinctly crystalline and are therefore more readily filterable.

A convenient form in which to use the phosphotungstic acid is that of a 25—30 % aqueous solution. Many samples of the acid recently purchased [1916] have been somewhat impure, and it is wise never to employ an acid, which fails to dissolve in water to a clear solution, until it has been purified by the ether process sufficiently to pass this test.

Demjanowski [1912] did not find any appreciable difference in the precipitability of creatinine, carnosine and methylguanidine when he employed 2·5 % and 25 % solutions of the reagent, so apparently the strength of the latter is a comparatively unimportant matter.

Re-solution of the precipitate upon adding an excess of the precipitating acid does not always occur. Many phosphotungstates are extremely readily soluble in such excess, whilst others are not so influenced. The condition of the precipitate itself is governed by several factors. Upon standing amorphous precipitates often become slowly crystalline, whilst in many cases slow precipitation from a very dilute solution of the base results in the production of a crystalline precipitate direct.

A modification of the latter process is valuable for obtaining crystals of a relatively large size, if such are desired for any purpose.

The crystalline form of phosphotungstic acid precipitates when derived from the amorphous forms is usually that of masses of thin needles or of short rhombohedral prisms. When however the crystals are allowed to form slowly, as described above, by precipitation from very dilute solution or when the precipitates are slowly recrystallised from suitable solvents it is sometimes possible to obtain specimens which are characteristic of the particular salt.

Unfortunately the number which yield such characteristic crystals is small, and it is only very occasionally that the crystalline form of the salt is any guide to the recognition of the parent base. Certain of the phosphotungstates examined during the course of this investigation possessed colour and in certain cases this is helpful for purposes of identification.

### 2. *Preparation of the purified phosphotungstates.*

The phosphotungstates were in all cases prepared by the addition of a very slight excess of 30 % phosphotungstic acid to a solution of the base or its salt in 5 % sulphuric acid. After standing for 24 hours the precipitates

were filtered off and well washed with 5 % sulphuric acid. The products thus obtained were submitted to an initial purification by recrystallisation from hot water and the crystalline salts dried over sulphuric acid in a darkened and evacuated desiccator. The storage of the pure products should be carried out in the absence of light since many of them tend to assume a blue or violet colour if exposed to photochemical influences.

When prepared by such a process the phosphotungstates are in the large majority of cases well defined, crystalline products. A few of them are coloured but the greater number are colourless. The crystalline form of the salts varies with the conditions of crystallisation. If this is rapid the crystals are usually thrown out of solution in thick felted masses of needles. On the contrary slow crystallisation produces well formed crystals which are in some cases sufficiently large to be examined with a pocket-lens.

The choice of a solvent for recrystallisation of the phosphotungstates can be made from water, ethyl alcohol, methyl alcohol, acetone or mixtures of two or more of these.

The experience gained during the present research has led to the adoption of water or 50 % ethyl alcohol as being the most suitable solvents for the purpose. Even when recrystallised from the same solvent the crystalline form of an individual phosphotungstate may on different occasions exhibit differences, so that the identification of any particular salt by this means is a somewhat untrustworthy process.

### 3. *Analysis and composition of the purified phosphotungstates.*

The purified salts obtained by the process just described were in a number of cases submitted to analysis. Moisture was determined by the usual process of drying to constant weight at 110°. Nitrogen was estimated by Kjeldahl. In some cases determinations of tungsten oxide were carried out. Various procedures for conducting the latter analysis were tested, the best results being obtained by the method of Barber [1906], also used with good results by Wechsler [1911]. Many of the phosphotungstates were submitted to analysis after more than one recrystallisation and it was generally found that their composition remained unaffected by the purification. Apparently some compounds are so affected, for Barber [1906] states that upon recrystallisation of glutaminic acid phosphotungstate isomorphous mixtures of the salt and the free acid are obtained.

### 4. *Determinations of Solubility.*

Determinations of the solubility of the individual phosphotungstates in various solvents were carried out where the amount of material permitted When this was not possible qualitative tests of this nature were made. The solubilities were estimated by determining the weight of salt dissolved in a known volume of solvent after they had remained in contact for 24 hours at room temperature with occasional agitation.

### 5. *Properties of the Individual Phosphotungstates.*

#### (*a*) *Amino-acids.*

*Glycine phosphotungstate,* was studied by Barber [1906], who prepared it by the addition of 50 % phosphotungstic acid solution to a saturated solution of the amino-acid. He describes it as crystallising in microscopic whetstone-shaped crystals. On attempting to dissolve it in warm water he found that tungstic acid was thrown out of solution.

Preparations dried at 105° gave upon analysis figures which agreed with the formula, $(C_2H_5ON_2)_3,H_3PO_4,12WO_3$. It normally occurs with water of crystallisation, approximately 5—6 molecules being present. Recrystallisation from hot alcohol results in glycine being deposited. Levene and Beatty [1906] record the precipitability of glycine by phosphotungstic acid.

*Alanine phosphotungstate* was described by Barber [1906] as crystallising in microscopic twin needles. It retains its composition after recrystallisation. Its analysis agrees with the formula $(C_3H_7O_2N)_3,H_3PO_4,12WO_3,4—5H_2O$. Levene and Beatty [1906] have studied its precipitability.

*Leucine phosphotungstate.* Barber [1906] could not prepare leucine phosphotungstate, and a similar failure was reported by Schulze and Winterstein [1901]. Levene and Beatty [1906], however, describe it as an oily product soluble in a small excess of the reagent.

*Aspartic acid phosphotungstate.* Barber [1906] states that it occurs in the form of stubby microscopic octahedra containing 24 molecules of water of crystallisation, and varying in composition, with the formula $(C_4H_7O_4N)_4$, $2H_3PO_4,20—22WO_3$. Its composition usually agrees most satisfactorily with $22WO_3$ and it is unchanged by recrystallisation. Levene and Beatty [1906] studied the precipitability of aspartic acid by phosphotungstic acid.

*Glutaminic acid phosphotungstate* is described by Barber [1906] as a flocculent mass which slowly becomes crystalline. Its composition as indicated

by the analytical figures indicates that it readily forms isomorphous mixtures with the free acid. Levene and Beatty [1906] describe it as slightly soluble in excess and they record its precipitability.

*Cystine phosphotungstate* was described by Winterstein [1901] who obtained a crystalline product. Plimmer [1913] has attempted to utilise the fact that cystine is completely precipitated from all but very dilute solutions by phosphotungstic acid as a basis for a method of separating cystine and tyrosine.

Cystine is readily precipitated from solution by means of phosphotungstic acid. The precipitate is usually of a pale cream colour and if thrown out of dilute solutions may be crystalline.

Upon recrystallisation from hot water, in which it is readily soluble, it separates out in very small rhombohedral tables of a pale sulphur yellow colour (Photograph 4). It does not alter in composition upon recrystallisation.

It usually contains 1·25 % water, equivalent to 4—5 molecules of water of crystallisation.

0·2041 g.; 17·6 cc. 0·01*N* $NH_3$ = 1·2 % N

0·2046 g.; 18·0 „ „ „ = 1·2 % N.

Calculated for $(C_6H_{12}O_4N_2S_2)_3, 2H_3PO_4, 24WO_3, 4—5H_2O$ = 1·28 % N.

It is very soluble in alcohol, methyl alcohol and acetone.

*Phenylalanine phosphotungstate* was described in the first place by Schulze and Winterstein [1901] as a pale yellow oil which becomes crystalline upon standing. They state that it is not very soluble in cold water and that it is not precipitated from dilute solutions of the amino-acid. Levene and Beatty [1906] record a similar description of the compound. For this investigation it was prepared from a strong solution of phenylalanine in 5 % sulphuric acid. It separates initially as a lemon yellow oil, sometimes possessing characteristic odour. Upon standing the oil becomes crystalline, forming masses of thin plates and needles. It is readily recrystallised from hot water and after separating as an oil again soon crystallises. The crystals are short rhombohedral prisms which on account of their colour are fairly characteristic. It contains approximately four molecules of water of crystallisation.

Upon analysis it gave the following figures:

0·2071 g.; 16·0 cc. 0·01 *N* $NH_3$ = 1·1 % N

0·2065 g.; 15·5 „ „ „ = 1·1 % N

0·1032 g.; 0·0855 g. $WO_3$ = 83·0 % $WO_3$.

Calculated for $(C_9H_{11}O_2N)_3, H_3PO_4, 12WO_3, 4H_2O$, N = 1·22 %, $WO_3$ 80·9 %.

Its composition is constant after repeated recrystallisation.

*Tyrosine phosphotungstate* was not obtained by Schulze and Winterstein [1901], nor by Barber [1906], moreover all attempts made during the course of this work to isolate the compound failed.

*Histidine phosphotungstate.* Wechsler [1911] has made an analysis of histidine phosphotungstate and considers that it is represented by the formula $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3$.

It is readily prepared as a white microcrystalline powder. Upon precipitation from very dilute solutions of the base or by recrystallisation from water or dilute alcohol, it may be obtained in comparatively large, clear, well-formed crystals consisting of rhombohedral prisms. (Photograph 3.)

Wechsler describes the substance as anhydrous. Specimens prepared during this investigation, however, gave on analysis indications of the presence of five molecules of water of crystallisation. Its composition remains unaltered with purification.

0·2083 g.; 3.45 cc. 0·1 *N* $NH_3$ = 2·3 % N
0·2059 g.; 3·20 cc. „ „ = 2·2 % N
0·1959 g.; 0·1708 g. $WO_3$ = 87·2 % $WO_3$
0·2170 g.; 0·1885 g. „ = 86·9 % $WO_3$.

Wechsler gives the following analysis of his product.

2·00 2·08 2·06 % N
86·4 89·8 — % $WO_3$.

Calculated for $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3$, N = 2·02 %, $WO_3$ = 89·3 %.
Calculated for $(C_6H_9O_2N_3)_3, 2H_3PO_4, 24WO_3, 5H_2O$, N = 1·98 %, $WO_3$ = 87·2 %.

The salt is very soluble in acetone and methyl alcohol but comparatively insoluble in water and alcohol.

*Arginine phosphotungstate.* Gulewitsch [1899] made a fairly complete study of this compound. Reference has already been made to his observations upon the conditions influencing its precipitation.

His preparations were submitted to analysis and gave the following data:

N 2·84 2·68 %
C 3·48 3·44 %
H 0·91 0·74 %.

From these figures he considered the substance was to be represented by

$$(C_6H_{14}O_2N_4)_3, 2H_3PO_4, 24WO_3, 10H_2O$$

which requires C = 3·46, N = 2·6, H = 0·65.

Wechsler [1911] agrees with this formula since his preparations contained 2·64 % and 2·70 % nitrogen.

Several preparations made during the course of this work were analysed, the following results being typical of those obtained.

0·2025 g.; 3·20 cc. 0·1 *N* $NH_3$ = 2·2 % N
0·2096 g.; 4·15 cc. „ „ = 2·8 % N
0·2288 g.; 0·2014 g. $WO_3$ = 88·0 % $WO_3$
0·2394 g.; 0·2104 g. „ = 87·9 % $WO_3$.

The substance contained water of crystallisation equivalent to 6—10 molecules.

Calculated for $(C_6H_{14}O_2N_4)_3, 2H_3PO_4, 24WO_3, 6H_2O$, N = 2·63 %, $WO_3$ = 87·2%.

The phosphotungstate prepared in the usual manner is a white amorphous powder. It may be readily obtained in the form of rhombohedral prisms of a comparatively large size by recrystallisation from water or dilute alcohol (Photograph 5). The salt tends to redissolve in the presence of a great excess of the reagent, a point also noted by Gulewitsch.

*Lysine phosphotungstate* is precipitated from great dilutions of the salts of the amino-acid as a white amorphous powder. It may be obtained in a crystalline condition by recrystallisation from dilute alcohol and usually separates out in large clusters of very fine needle-shaped crystals. It retains its composition although subjected to many recrystallisations and is anhydrous. Upon analysis it yields figures which satisfy the formula

$(C_6H_{14}O_2N_2)_3, 2H_3PO_4, 24WO_3$.

0·2089 g.; 18·0 cc. 0·01 *N* $NH_3$ = 1·2 % N
0·2094 g.; 19·0 cc. „ „ = 1·3 % N.
Required for above formula 1·35 % N.

No other amino-acid phosphotungstates were investigated in this research. The solubilities of those whose other properties have been described are given in the table of solubilities at the end of the paper.

### (*b*) *Amines.*

The basic nature possessed by representatives of this class of compound is responsible for the formation of a series of well-defined salts with phosphotungstic acid. Generally speaking they are precipitated from very dilute solutions of the parent base as heavy insoluble microcrystalline powders. One or two of them possess colour which renders their identification possible.

*Trimethylamine phosphotungstate* is precipitated from exceedingly dilute solutions of the base as a heavy white amorphous powder. It may be obtained

in the form of microscopic octahedra by recrystallisation from water. Its composition agrees with the formula $((CH_3)_3N)_3, H_3PO_4, 12WO_3$.

Analysis: 0·2011 g.; 18·9 cc. 0·01 *N* $NH_3 = 1·3$ % N.

Calculated for above formula = 1·37 % N.

It is readily soluble in acetone but not appreciably soluble in the other solvents examined.

*Isobutylamine phosphotungstate* separates out as a heavy white flocculent precipitate from solutions of the base upon the addition of phosphotungstic acid. It is very soluble in acetone, but not greatly so in water or ethyl alcohol. It crystallises in large clear rhombohedral prisms.

*Isoamylamine phosphotungstate* is closely similar to the preceding compound in its crystalline appearance and solubilities.

*Tri-isobutylamine, Tripropylamine and Tri-isoamylamine phosphotungstates* are closely similar to each other. They are all precipitated from great dilution as heavy white, amorphous precipitates. They crystallise in microcrystalline rhombohedral prisms and tablets, and occasionally in fern-like aggregates closely resembling those assumed by ammonium chloride.

Their solubilities are similar, all being exceedingly soluble in acetone or dilute acetone, practically insoluble in water and ethyl alcohol and slightly soluble in methyl alcohol. The composition of two of these salts was investigated.

*Tri-isobutylamine phosphotungstate.*

0·4699 g. anhydrous salt; 4·4 cc. 0·1 *N* $NH_3 = 1·3$ % N.

Required for $(C_{12}H_{27}N)_3, H_3PO_4, 12WO_3$ = 1·23 % N.

*Tri-isoamylamine phosphotungstate.*

0·5832 g. anhydrous salt; 3·6 cc. 0·1 *N* $NH_3 = 1·4$ % N.

Required for $(C_8H_{11}N)_3, H_3PO_4, 12WO_3$ = 1·3 % N.

*Putrescine and Cadaverine phosphotungstates.* Putrescine and cadaverine are both precipitated from very dilute solution by phosphotungstic acid. The resulting salts are heavy white amorphous powders which can be obtained in a microcrystalline condition by recrystallisation from water or alcohol. The crystals are not characteristic, being as is the case with the majority of these salts, rhombohedral prisms.

They exhibit similar solubilities to one another. Acetone or dilute acetone readily dissolves them, but in water they are practically insoluble. Ethyl and methyl alcohol will take up a fair amount of the salts.

*Phenylethylamine phosphotungstate.* This compound closely resembles the

related phenylalanine phosphotungstate in many of its properties. It is not precipitated from dilute solutions of the base except on long standing. It separates out initially as a pale sulphur yellow oil which slowly becomes crystalline. On recrystallisation from water the coloured oil once again separates but the crystals which subsequently form are fairly large and well-formed. They usually appear as pale yellow prisms and tablets, closely resembling the salt of phenylalanine.

The analysis of the purified anhydrous salt gave the data:

0·3517 g.; 3·6 cc. 0·1 *N* $NH_3$ = 1·4 % N.

Required for $(C_8H_{11}N)_3, H_3PO_4, 12WO_3$ = 1·3 % N.

Its composition is unaltered by repeated purification. Its solubilities are given in the table on p. 22.

p-*Hydroxyphenylethylamine phosphotungstate* is not precipitated from dilute solutions except on long standing. From concentrated solutions it is thrown out as a straw coloured microcrystalline powder, consisting of flat cubical plates. When deposition occurs slowly or when recrystallisation of the product is carried out with care the compound is readily obtained in the form of large orange-red octahedra, the colour of which resembles that of potassium dichromate.

The salt obtained by these methods is anhydrous and its composition corresponds to the formula $(C_8H_{11}ON)_3, H_3PO_4, 12WO_3$.

0·2190 g.; 2·05 cc. 0·1 *N* $NH_3$ = 1·3 % N.

Required for above formula = 1·28 % N.

It is not very soluble in cold water but readily in hot. It is fairly soluble in ethyl alcohol and very soluble in acetone. The crystalline form and colour of this salt are of help in its identification.

*β-Iminazolyl-ethylamine phosphotungstate.* This compound is precipitated from great dilutions as is the corresponding amino-acid compound. It separates out as a white microcrystalline powder which often assumes a beautifully crystalline form upon standing. These crystals may also be obtained by recrystallisation from water or dilute alcohol, the salt separating from these solvents in the form of relatively large clear rhombohedra and prisms. Its composition agrees with the formula $(C_5H_9N_3)_3, 2H_3PO_4, 24WO_3$. Its solubilities and other properties are very similar to those of histidine phosphotungstate.

*Indolethylamine phosphotungstate.* This salt is precipitated from very dilute solutions of the base as a terra cotta coloured amorphous powder. Upon standing this undergoes change and becomes crystalline forming large

aggregates of needles possessed of a deep brownish purple colour. Upon recrystallisation the amorphous form usually separates out first, the transition to the crystalline form occurring sooner or later upon standing. The microscopical appearance of the crystals of the salt is characteristic.

Upon analysis the following figures for the determination of nitrogen were obtained.

0·1771 g.; 3·5 cc. 0·1 *N* $NH_3$ = 2·8 % N.
Required for $(C_{10}H_{12}N_2)_3, H_3PO_4, 12WO_3$ = 2·5 % N.

The compound is very soluble in acetone and in diluted acetone, and fairly soluble in ethyl and methyl alcohol but is only very slightly soluble in cold water.

(*c*) *Purine Bases.*

The purine bases, generally speaking, are precipitated from very dilute solutions by phosphotungstic acid and the resulting salts are peculiar in being very slightly soluble, even in acetone. They crystallise in cubical plates and may in some cases be recognised by this microscopical appearance. Determinations of nitrogen by the Kjeldahl process do not yield accurate results since the purine nitrogen is not quantitatively accounted for by that method.

*Adenine phosphotungstate* is usually precipitated as a pale cream coloured powder. It is readily obtained in the form of clear sulphur-yellow cubical, or whetstone-shaped, crystals by careful recrystallisation from dilute alcohol or acetone (Photograph 2). It is very insoluble in water, and ethyl and methyl alcohols and only very slightly soluble in acetone. Funk [1915] has isolated adenine from the acetone-insoluble fraction of the phosphotungstates from autolysed yeast.

Analysis:

0·1050 g.; 0·0899 g. $WO_3$; 85·6 % $WO_3$.
Required for $(C_5H_5N_5)_3, H_3PO_4, 12WO_3$; 86·0 % $WO_3$.

*Guanine phosphotungstate* is precipitated from extremely dilute solutions of guanine in the form of a buff coloured amorphous precipitate. Wechsler [1911] states that it possesses a brick red colour but the specimens prepared from pure guanine during this investigation were much paler in colour than he describes. Recrystallised from water or dilute alcohol it forms bunches of straw coloured, needle-shaped crystals or small cubical plates. Its properties are closely similar to those of the corresponding adenine salt.

*Hypoxanthine phosphotungstate* is precipitated as a pale cream coloured amorphous precipitate. It crystallises in small cubical plates possessing a

light straw colour. Its solubilities are very similar to those of adenine and guanine.

*Xanthine phosphotungstate* is very similar in its properties and its crystalline appearance to the hypoxanthine salt.

*Uric acid phosphotungstate.* The phosphotungstate of uric acid is referred to by Hammarsten [1909, p. 672] as a brick red coloured powder.

It is prepared by precipitation of a solution of sodium urate by phosphotungstic acid and forms a deep chocolate brown powder. It cannot be recrystallised from water or alcohol owing to formation of insoluble uric acid. If prepared from hot sodium urate solutions it may be obtained in a crystalline condition, the crystals being cubical plates and of a deep red brown colour.

### (*d*) *Betaïnes and other Bases.*

The betaïnes form well defined crystalline salts with phosphotungstic acid as also do many of the naturally occurring bases. The representatives of both classes are numerous and only a few which were readily obtainable were examined.

*Betaïne phosphotungstate.* Betaïne is precipitated from very dilute solutions by phosphotungstic acid as a heavy white precipitate. The salt may be obtained in the form of large clear rhombohedral prisms by recrystallisation from water or dilute alcohol (Photograph 1). It is exceedingly soluble in acetone and in dilute acetone. Its composition satisfies the formula

$$(C_5H_{11}O_2N)_3, H_3PO_4, 12WO_3.$$

0·3889 g.; 32·2 cc. 0·01 *N* $NH_3$ = 1·2 % N
0·3893 g.; 36·95 cc. „ „ = 1·3 % N
0·4658 g.; 0·4040 g. $WO_3$ = 86·7 % $WO_3$.

Required for above formula N = 1·3 %, $WO_3$ = 86·2 %.

Repeated recrystallisation does not affect its composition.

*Stachydrine phosphotungstate.* Solutions of stachydrine salts yield a heavy, white granular precipitate with phosphotungstic acid. The salt after purification by recrystallisation forms clusters of needles and small rhombohedral prisms, often with cut edges. It is very soluble in acetone and dilute acetone. It is anhydrous and its composition agrees with the formula

$$(C_7H_{13}O_2N)_3, H_3PO_4, 12WO_3.$$

*Nicotinic acid phosphotungstate* is very insoluble and is therefore precipitated from great dilutions of the base. It can be obtained in a crystalline condition forming small rhombohedral prisms. It is fairly soluble in acetone.

*Choline phosphotungstate.* Choline is precipitated from very dilute solutions (Gulewitsch states 1 in 20,000) by phosphotungstic acid. The initial precipitate is a heavy white amorphous substance. It can be obtained in a micro-crystalline form by recrystallisation from dilute alcohol. The crystals are minute rhombohedral prisms frequently possessing faceted edges.

Analysis of the purified substance:

0·3884 g.; 36·0 cc. 0·01 *N* $NH_3$ = 1·3 % N
0·3869 g.; 37·2 cc. „ „ = 1·4 % N.
Required for $(C_5H_{13}O_2N)_3, H_3PO_4, 12WO_3$ = 1·29 % N.

The salt remains unchanged in composition after more than one recrystallisation. It is readily soluble in acetone and dilute acetone but in other solvents is very insoluble.

Drummond and Funk [1914] record finding choline in the acetone-soluble fraction of the phosphotungstates from rice polishings.

*Colamine (amino-ethyl alcohol) phosphotungstate.* Barger [1914] records that colamine is not precipitated by phosphotungstic acid unless the solutions of the base are concentrated. This was found to be the case and the salt itself is very liable to re-solution in an excess of the reagent. Recrystallised from water or alcohol it forms beautiful clear rhombohedral crystals which often attain a relatively large size. The separation of choline and colamine by a method depending on the solubilities of their phosphotungstates is practicable. The latter salt is very soluble in water, alcohol or acetone.

*Urea phosphotungstate.* Urea is precipitated by phosphotungstic acid from its solutions, if these are not too dilute. The resulting white amorphous salt may be readily recrystallised from hot water in which it is very soluble. Large well-formed rhombohedral prisms are thus obtained which are easy to recognise (Photograph 6). The salt is readily soluble in ethyl and methyl alcohols and in acetone.

0·2002 g.; 3·4 cc. 0·1 *N* $NH_3$ = 2·3 % N
0·2071 g.; 4·01 cc. „ „ = 2·7 % N.
Required for $(CH_4ON_2)_3, H_3PO_4, 12WO_3$ = 2·74 % N.

Its composition is unaltered by repeated recrystallisation.

*Creatinine phosphotungstate.* Dilute solutions of creatinine do not always give a precipitate immediately upon the addition of phosphotungstic acid. Upon standing, however, the crystalline salt is in many cases slowly deposited. Hofmeister [1880] gives the limit of precipitability as 1 in 12,000 whilst Demjanowski [1912] obtained precipitation at a dilution of 1 in 25,000. Precipitated in this manner from dilute solutions of the base the phospho-

tungstate may be obtained in large well-formed crystals which are fairly characteristic. They usually consist of rhombohedra and rhombohedral prisms. When precipitated from more concentrated solutions the salt is thrown down as a heavy white precipitate consisting of microscopic needle-shaped crystals. The pure salt is anhydrous and is not very soluble in water or alcohol but is very soluble in various dilutions of acetone.

Analysis:

0·2090 g.; 5·91 cc. 0·1 *N* $NH_3$ = 4·0 % N
0·2070 g.; 5·59 cc. „ „ = 4·0 % N
0·1002 g.; 0·0865 g. $WO_3$ = 86·3 % $WO_3$.

Required for $(C_4H_7ON_3)_3, H_3PO_4, 12WO_3$ = N 3·92 %, $WO_3$ 86·7 %.

Its composition is unaltered by repeated recrystallisation.

*Carnosine phosphotungstate.* Carnosine is precipitated from very dilute solutions as the phosphotungstate in the form of a white heavy powder. It recrystallises in very minute whetstone- and needle-shaped crystals from water or dilute alcohol. Demjanowski [1912] describes its crystalline form and records its precipitability.

Analysis:

0·2074 g.; 3·47 cc. 0·1 *N* $NH_3$ = 2·3 % N
0·2082 g.; 3·60 cc. „ „ = 2·4 % N.

Required for $(C_9H_{14}O_3N_4)_3, 2H_3PO_4, 24WO_3$ = 2·6 % N.

Its composition is unaltered by purification It is very soluble in acetone and dilute acetone.

*Guanidine phosphotungstate* is very insoluble in water and is precipitated from very dilute solutions of guanidine. When recrystallised from water or dilute alcohol it usually separates out again as a white microcrystalline powder consisting of minute spherulites of needles. Small rhombohedra are also sometimes obtained.

0·3904 g.; 11·49 cc. 0·1 *N* $NH_3$ = 4·1 % N
0·3879 g.; 11·36 cc. „ „ = 4·1 % N
0·3715 g.; 0·3461 g. $WO_3$ = 93·2 % $WO_3$.

Required for $(CH_5N_3)_3, H_3PO_4, 12WO_3$ = 4·16 % N and 90·5 % $WO_3$.

It is very soluble in acetone and mixtures of acetone and water.

*Methylguanidine phosphotungstate* is a heavy white powder which by recrystallisation from water or alcohol yields crystals consisting of small rhombohedral prisms with cut edges. Demjanowski [1912] describes a similar appearance for the salt. It is precipitated from very dilute solutions of methylguanidine salts.

### (*e*) *Inorganic Phosphotungstates.*

A few inorganic salts of phosphotungstic acid are of importance from the point of view of this research, since the inorganic bases they contain are frequently present in tissue extracts or other material under investigation. The most important of these salts are the phosphotungstates of sodium, potassium and ammonium.

*Ammonium, Sodium and Potassium phosphotungstates.* Sodium phosphotungstate is very soluble in water and can only be obtained by concentration of its solutions almost to dryness.

Potassium phosphotungstate on the other hand is very slightly soluble in water and still less so in 5 % sulphuric acid, so that sodium and potassium may be almost quantitatively separated by this means. Ammonium phosphotungstate exhibits similar solubilities to the potassium salt, being very slightly soluble in most solvents. They are both difficult to obtain in a crystalline condition, usually occurring as very fine white precipitates which readily pass through filters.

### *Solubilities of the Phosphotungstates.*

The solubilities in a number of solvents of all the phosphotungstates prepared during the course of this investigation have been determined, quantitatively where possible, and qualitatively where the amount of material was insufficient for the purpose, and the results are summarised in Table III.

The determinations were in all cases carried out at room temperature (18—20° C.) and are expressed in grams per 100 g. of solvent.

The figures given for the solubilities of certain of the phosphotungstates in 57 % acetone are taken from the paper by Wechsler [1911].

The original aim of the investigation, as has already been stated, was to attempt the separation of mixtures of bases by taking advantage of differences in the solubilities of their phosphotungstates. Wechsler compared the solubilities of a number of these salts in a solvent composed of four volumes of acetone to three of water and concluded that his results had a practical value. Reviewing the table of solubilities we see that wide variations in solubility are few.

The relatively great solubility of the majority of phosphotungstates in acetone is marked and this solubility becomes much greater if water is present.

The following series of solubility determinations in various dilutions of acetone were made upon a preparation of adenine phosphotungstate.

TABLE III

| Phosphotungstate | Water | Ethyl alcohol | 50% Ethyl alcohol | Methyl alcohol | Acetone | 75 % Acetone | 57 % Acetone | 50 % Acetone | 25 % Acetone | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| Glycine ... ... ... | 4·5 | 14·44 | 21·30 | v.s. | v.s. | v.s. | — | v.s. | v.s. | — |
| Alanine ... ... ... | 8·38 | 60·0 | 400·0 | v.s. | v.s. | v.s. | — | v.s. | v.s. | — |
| Aspartic Acid ... ... | 3·0 | 23·8 | 400·0 | v.s. | v.s. | v.s. | — | v.s. | v.s. | — |
| Glutaminic Acid ... ... | f.s. | f.s. | — | v.s. | v.s. | — | — | — | — | — |
| Cystine ... ... ... | sl.s. cold, v.s. hot | sl.s. cold, v.s. hot | — | v.s. | v.s. | v.s. | — | v.s. | v.s. | — |
| Phenylalanine ... ... | f.s. cold | f.s. | — | v.s. | v.s. | v.s. | — | v.s. | v.s. | — |
| Histidine ... ... ... | 0·4 | 8·8 | v.s. | 30·6 | 33·4 | v.s. | 160·0 | v.s. | v.s. | Insoluble in ether, $CHCl_3$, $CCl_4$ |
| Arginine ... ... ... | 0·2 | 3·0 | v.s. | 28·7 | 25·6 | v.s. | 120–130 | v.s. | v.s. | ,, ,, |
| Lysine ... ... ... | 0·3 | 3·6 | f.s. | 9·6 | 12·6 | v.s. | 140 | v.s. | v.s. | ,, ,, |
| Trimethylamine ... ... | v.sl.s. | v.sl.s. | sl.s. | sl.s. | v.s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Isobutylamine ... ... | v.sl.s. | sl.s. | f.s. | f.s. | v.s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Isoamylamine ... ... | v.sl.s. | v.sl.s. | f.s. | f.s. | v.s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Tripropylamine ... ... | v.sl.s. | v.sl.s. | f.s. | sl.s. | v.s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Triamylamine ... ... | v.sl.s. | sl.s. | f.s. | f.s. | v.s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Putrescine ... ... ... | v.sl.s. | f.s. | — | f.s. | v s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Cadaverine ... ... ... | v.sl.s. | sl.s. | 2 | sl.s. | v.s. | v.s. | — | v.s. | v.s. | ,, ,, |
| Phenylethylamine ... ... | sl.s. | f.s. | — | f.s. | v.s. | — | — | v.s. | — | ,, ,, |
| *p*-Hydroxyphenylethylamine | sl.s. | f.s. | — | v.s. | v.s. | — | — | v.s. | — | ,, ,, |
| *β*-Iminazolyl-ethylamine ... | sl.s. | f.s. | — | f.s. | v.s. | — | — | v.s. | — | ,, ,, |
| Indolethylamine ... ... | v.sl.s. | f.s. | — | v.s. | v.s. | — | — | v.s. | — | ,, ,, |
| Adenine ... ... ... | 0·05 | 0·05 | 0·5 | 0·1 | 2·9 | 19·3 | 10·5 | 9·4 | 0·1 | ,, ,, |
| Guanine ... ... ... | 0·05 | 0·08 | — | 1·0 | 2·2 | — | 7·21 | 9·0 | — | ,, ,, |
| Xanthine ... ... ... | v.sl.s. | v.sl.s. | — | sl.s. | sl.s. | — | — | f.s. | — | ,, ,, |
| Hypoxanthine ... ... | v.sl.s. | v.sl.s. | — | sl.s. | sl.s. | — | — | f.s. | — | ,, ,, |
| Urea ... ... ... ... | v.s. | v.s. | v.s. | v.s. | v.s. | — | — | v.s. | — | — |
| Creatinine ... ... ... | 0·2 | 0·3 | 2·7 | 0·6 | 8·0 | v.s. | 13·34 | 21·5 | — | — |
| Carnosine ... ... ... | v.sl.s. | sl.s. | — | f.s. | v.s. | v.s. | — | v.s. | — | — |
| Guanidine ... ... ... | 0·2 | 0·4 | 19·5 | 29·2 | 9·9 | v.s. | 22·8 | 29·7 | 27·1 | Insoluble in ether, $CHCl_3$ or $CCl_4$ |
| Methylguanidine ... ... | v.sl.s. | sl.s. | — | sl.s. | f.s. | — | — | v.s. | — | ,, ,, |
| Colamine ... ... ... | v.s. | v.s. | — | v.s. | v.s. | — | — | v.s. | — | ,, ,, |
| Betaine ... ... ... | 0·1 | 0·1 | 2·8 | 1·2 | 30·5 | v.s. | — | v.s. | v.s. | ,, ,, |
| Choline ... ... ... | 0·02 | 0·02 | 0·5 | 0·6 | 115·0 | 37·0 | — | 16·8 | 0·6 | ,, ,, |
| Stachydrine ... ... | 0·05 | 0·03 | 0·8 | — | 23·0 | v.s. | — | v.s. | 3·4 | ,, ,, |
| Nicotinic acid ... ... | 0·05 | 0·20 | 2·1 | — | 6·5 | 58·0 | — | 12·9 | 3·5 | ,, ,, |
| Sodium ... ... ... | v.s. | v.s. | — | — | — | — | — | — | — | ,, ,, |
| Potassium ... ... ... | 0·3 | 0·3 | — | 4·5 | 0·3 | — | — | v.sl.s. | — | ,, ,, |
| Ammonium ... ... ... | 0·2 | 0·2 | — | 3·0 | 0·2 | — | — | v.sl.s. | — | ,, ,, |

v. = very. sl. = slight. f. = fairly.

TABLE IV.

| % volume acetone | Solubility |
|---|---|
| 100 | 2·9 |
| 80 | 15·4 |
| 75 | 19·3 |
| 60 | 12·3 |
| 60 | 9·4 |
| 30 | 2·0 |
| 25 | 0·1 |
| 10 | 0·07 |
| 0 | 0·05 |

The greatest solubility usually occurs, as in the above case, when acetone of about 90—75 % is used.

Acetone has already been used as a means of separating phosphotungstic acid precipitates into fractions, Funk [1915] and Drummond and Funk [1914]. The purines are usually found in the acetone-insoluble fractions, an observation which is in agreement with the solubility data recorded here.

The question of the practical utility of these results is an open one and it must be left so until experience has shown their value. The separation of sodium and potassium phosphotungstates, taking advantage of the great difference in their solubilities in water, the separation of the purine bases by the insolubility of their phosphotungstates in acetone, the separation of cystine and tyrosine, a process already examined by Plimmer [1913], and the separation of choline and colamine are a few of the more obvious applications of these results.

In the earlier stages of the work it was believed that the phosphotungstates of certain substances might be used as a help to the identification of the parent base. Unfortunately this hope is now much shaken. Few of the salts examined are sufficiently characteristic in their appearance to admit of easy recognition, whilst their composition renders the value of analytical data somewhat doubtful.

Certain phosphotungstates are fairly characteristic, particularly those which possess colour. Such for example are those of adenine, guanine, uric acid, *p*-hydroxyphenylethylamine, indolethylamine, phenylalanine, phenylethylamine and cystine. Others such as creatinine, urea, and colamine may in some cases be recognised by their crystalline form. Photomicrographs of a few of the crystalline phosphotungstates are given at the end of this paper (Plate I).

The recrystallisation of mixtures of purified phosphotungstates with the

object of identifying the individual components has yielded very disappointing results since the tendency to form mixed crystals is marked.

I must express my gratitude and indebtedness to Dr O. Rosenheim and Dr A. J. Ewins for their kindness in placing at my disposal pure specimens of many of the bases which have been examined during the course of this work.

## Summary.

1. A large number of the salts formed by amino-acids and nitrogenous bases with phosphotungstic acid have been prepared in a pure condition.

2. The composition, crystalline form and solubilities of these compounds have been examined.

3. In a few isolated cases the crystalline form or colour of the purified salt gives an indication as to the identity of the parent base, whilst separations based upon different solubilities of the salts may occasionally be made.

4. The optimum conditions for the precipitation of bases by phosphotungstic acid have been examined. The results confirm the observations of Gulewitsch that a slight excess of the precipitant operating in a medium of 5 % sulphuric acid yields the best results.

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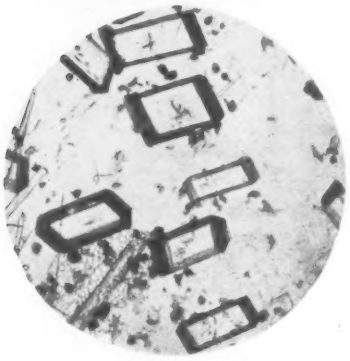
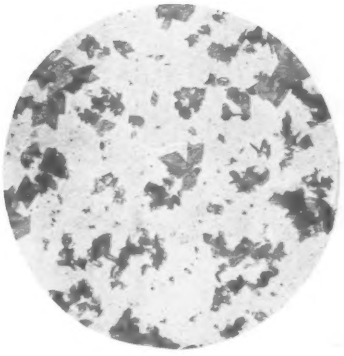


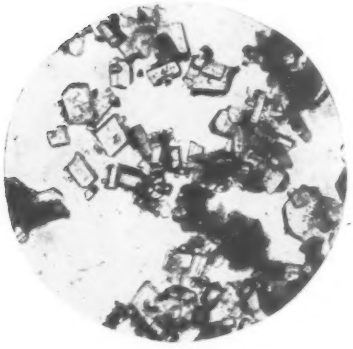
1. Betaïne Phosphotungstate.



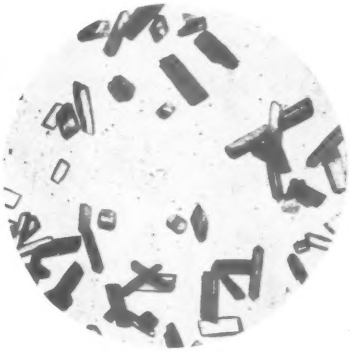
4. Cystine Phosphotungstate.

2. Adenine Phosphotungstate.

5. Arginine Phosphotungstate.



3. Histidine Phosphotungstate.



6. Urea Phosphotungstate.

Photomicrographs of some crystalline Phosphotungstates
(utilising 4 mm. objective and ocular 4. (Leitz.))

# II. A STUDY OF THE WATER-SOLUBLE ACCESSORY GROWTH PROMOTING SUBSTANCE. II. ITS INFLUENCE UPON THE NUTRITION, AND NITROGEN METABOLISM OF THE RAT.

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(*Received Jan. 30th, 1918.*)

In a previous communication it was shown that certain properties of the water-soluble accessory substance are similar to those which are associated with the so-called "anti-neuritic vitamine" [Drummond, 1917]. This fact, taken into consideration with the observation of McCollum and Kennedy [1916] that many extracts known to contain the water-soluble growth factor are active in the prevention or cure of polyneuritis in birds, led to the suggestion being advanced that the two substances are identical. If this view is correct, it cannot but be apparent from recent research how very important a rôle is played by this dietary factor in the nutrition of the animal organism. The present paper presents a series of observations bearing more or less directly upon this subject, and although they do not represent the results of a systematic research, they have been collected together in the hope that they may assist in throwing light upon certain aspects of the question.

### 1. *Influence of a deficiency of the water-soluble accessory factor in the diet upon the nitrogen metabolism of the rat.*

Four healthy young male rats weighing approximately 90–100 grams each were selected for this study. They were placed in pairs in metabolism cages of the type designed by Dr Alexander Paine, Director of this Institute. Modifications of this apparatus have been described [Ackroyd and Hopkins, 1916], but the original form was found to satisfy the requirements of the present investigation. By preparing the dietaries in the form of stiff pastes, the animals are prevented from scattering the food about to any appreciable

extent. Fresh food and water were supplied every morning, the food-consumption being estimated by weighing the food containers before and after every 24 hour period. The 24 hours' urine was collected in a 100 cc. Erlenmeyer flask containing a few cc. of very dilute acetic acid. This, together with the cage washings, was filtered through glass-wool in order to remove fur and other extraneous matter, and made up to a known volume, usually 50 cc. The estimations were made by the usual methods; total nitrogen in faeces and urine by micro-Kjeldahl, urea by urease, ammonia by the aeration method of Folin, and creatinine and creatine by a modification of the micro-method used by Folin and Morris [1913] in their investigation of the normal protein metabolism of the rat. Two animals were fed throughout upon an artificial ration (Diet 1) which has been extensively utilised in this laboratory, and which is known to be adequate as regards all the known dietary components, since three generations of rats have been reared to maturity in the normal period upon it. The other two rats were fed for a short preliminary period upon the complete ration; then, when it had been ascertained that they were responding with a normal rate of growth, they were given Diet 2, which resembled Diet 1 in every way except for the absence of the water-soluble factor (yeast extract). The composition of the diets was as given below:

| | Diet 1 | Diet 2 |
|---|---|---|
| Caseinogen ... | 18 parts | 18 parts |
| Butter-fat ... | 20 „ | 20 „ |
| Starch ... ... | 46 „ | 52 „ |
| Agar ... ... | 5 „ | 5 „ |
| Salt mixture ... | 5 „ | 5 „ |
| Yeast extract... | 6 „ | 0 „ |

In order to render the diets of a pasty consistency a small quantity of distilled water was added in each case.

The tabulated results of the metabolism experiments are given in Tables I and II. These results will now be considered in detail, and other observations bearing upon each subject will be introduced where appropriate.

(*a*) *Nitrogen Metabolism.* The urinary analyses tabulated in Tables I and II do not show any striking abnormality, other than the appearance of creatinuria during the period upon the diet deficient in the water-soluble substance. This is not surprising in view of the slow decline in body-weight of the rats upon this ration caused by wasting of the skeletal muscles observed

TABLE I.

*Metabolism of two normal rats fed upon a complete artificial Dietary. (Diet 1.)*

| Day | Body-weights g. | Rectal temperature °C. | Food consumed g. | N intake g. | Calorific intake | Total N in urine g. | % urea N | % ammonia N | % creatinine N | % creatine + creatinine N | N in faeces g. | Total N excreted g. | N balance g. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 94 118 | 38·4 38·2 | 31·0 | 0·861 | 117 | 0·491 | — | — | — | — | 0·050 | 0·541 | +0·320 |
| 2 | — | — | 32·6 | 0·904 | 123 | 0·603 | — | — | — | — | 0·041 | 0·644 | +0·260 |
| 3 | — | — | 26·2 | 0·665 | 99 | 0·251 | 80·0 | 11·5 | 0·65 | 0·70 | 0·046 | 0·297 | +0·368 |
| 4 | — | — | 21·1 | 0·538 | 80 | 0·234 | — | — | — | — | 0·036 | 0·270 | +0·268 |
| 5 | 109 131 | 38·4 37·9 | 22·5 | 0·571 | 85 | 0·210 | 76·7 | 11·1 | 1·03 | 1·04 | 0·042 | 0·252 | +0·319 |
| 6 | — | — | 23·6 | 0·600 | 89 | 0·233 | 77·7 | 12·0 | 1·03 | 1·03 | 0·033 | 0·266 | +0·334 |
| 7 | 120 141 | — | 33·3 | 0·846 | 126 | 0·255 | — | — | — | — | 0·027 | 0·282 | +0·564 |
| 8 | — | — | 29·3 | 0·731 | 111 | 0·320 | 79·2 | 8·5 | 0·81 | 0·86 | 0·043 | 0·363 | +0·368 |
| 9 | — | — | 32·9 | 0·822 | 124 | 0·307 | 80·1 | 10·0 | 0·94 | 0·99 | 0·047 | 0·354 | +0·468 |
| 10 | — | — | 34·6 | 0·865 | 131 | 0·318 | 76·9 | 8·2 | 0·92 | 0·96 | 0·038 | 0·356 | +0·509 |
| 11 | 128 151 | 38·4 38·2 | 37·7 | 0·943 | 143 | 0·384 | — | — | — | — | 0·055 | 0·439 | +0·504 |
| 12 | — | — | 35·2 | 0·880 | 132 | 0·425 | — | — | — | — | 0·049 | 0·474 | +0·406 |
| 13 | — | — | 28·4 | 0·710 | 107 | 0·396 | — | — | — | — | 0·048 | 0·444 | +0·266 |
| 14 | 137 160 | 38·4 38·3 | 29·8 | 0·745 | 113 | 0·421 | 80·2 | 8·5 | 0·72 | 0·79 | 0·043 | 0·464 | +0·281 |
| 15 | — | — | 35·8 | 0·902 | 136 | 0·406 | 80·1 | 8·6 | 0·95 | 0·96 | 0·053 | 0·459 | +0·443 |
| 16 | — | — | 29·4 | 0·740 | 111 | 0·380 | — | — | — | — | 0·041 | 0·421 | +0·319 |
| 17 | 149 169 | — | 34·8 | 0·878 | 132 | 0·356 | — | — | — | — | 0·047 | 0·403 | +0·475 |
| 18 | — | — | 36·8 | 0·928 | 139 | 0·478 | 78·2 | 10·0 | 1·12 | 1·16 | 0·030 | 0·508 | +0·420 |
| 19 | 157 178 | 38·4 38·7 | 38·6 | 0·972 | 146 | 0·529 | 78·9 | 8·9 | 1·03 | 1·03 | 0·047 | 0·576 | +0·396 |

## TABLE II.

*Metabolism of two normal rats upon a diet deficient in water-soluble substance.* (*Diet* 2.)

(*Fore and after periods on Diet* 1.)

| Day | Diet | Body-weights g. | | Rectal temperatures °C. | | Remarks | Food consumed g. | N intake g. | Caloric intake | Total N in urine g. | % urea N | % ammonia N | % creatinine N | % creatine + creatinine N | N in faeces g. | Total N excreted g. | N balance g. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 84 | 86 | 38·0 | 37·9 | — | 42·5 | 1·300 | 160 | 0·315 | 73·5 | 10·0 | 0·94 | 0·98 | 0·052 | 0·367 | +0·933 |
| 2 | 1 | — | | — | | — | 28·1 | 0·840 | 107 | 0·310 | — | — | — | — | 0·033 | 0·343 | +0·497 |
| 3 | 1 | 93 | 101 | — | | Blood examination normal ... | 29·5 | 0·900 | 112 | 0·284 | 82·9 | 9·1 | 0·76 | 0·78 | 0·054 | 0·338 | +0·562 |
| 4 | 1 | — | | — | | — | 31·5 | 0·950 | 119 | 0·252 | — | — | — | — | 0·044 | 0·296 | +0·654 |
| 5 | 1 | 106 | 116 | 38·1 | 38·0 | — | 27·7 | 0·835 | 105 | 0·262 | 82·1 | 7·3 | 1·08 | 1·08 | 0·023 | 0·285 | +0·550 |
| 6 | 1 | — | | — | | Animals very fit ... ... | 20·5 | 0·610 | 82 | 0·232 | — | — | — | — | 0·016 | 0·248 | +0·362 |
| 7 | 2 | 109 | 119 | 38·3 | 38·3 | — | 12·1 | 0·233 | 46 | 0·285 | — | — | — | — | 0·022 | 0·307 | −0·174 |
| 8 | 2 | — | | — | | — | 8·5 | 0·157 | 32 | 0·175 | — | — | — | — | 0·026 | 0·201 | −0·044 |
| 9 | 2 | 107 | 110 | 38·2 | 38·5 | — | 17·6 | 0·356 | 70 | 0·136 | 80·7 | 6·2 | 0·63 | 0·90 | 0·047 | 0·201 | +0·155 |
| 10 | 2 | — | | — | | Urine dark ... ... ... | 14·5 | 0·290 | 57 | 0·171 | 75·3 | 8·2 | 1·12 | 1·32 | 0·020 | 0·191 | +0·100 |
| 11 | 2 | — | | — | | „ ... ... ... | 17·8 | 0·340 | 71 | 0·182 | — | — | — | — | 0·016 | 0·198 | +0·142 |
| 12 | 2 | — | | — | | — | 14·0 | 0·252 | 56 | 0·171 | — | — | 1·19 | 1·36 | 0·040 | 0·210 | +0·042 |
| 13 | 2 | 106 | 107 | 38·0 | 37·7 | Urine dark ... ... ... | 15·1 | 0·282 | 60 | 0·198 | — | — | — | — | 0·026 | 0·224 | +0·058 |
| 14 | 2 | — | | — | | „ ... ... ... | 14·1 | 0·264 | 56 | 0·209 | — | — | — | — | 0·022 | 0·231 | +0·033 |
| 15 | 2 | — | | — | | Urine scanty ... ... ... | 13·4 | 0·249 | 53 | 0·192 | — | — | — | — | 0·041 | 0·233 | +0·016 |
| 16 | 2 | — | | — | | „ ... ... ... | 12·8 | 0·236 | 52 | 0·195 | 74·5 | 7·4 | 0·97 | 1·21 | 0·025 | 0·220 | +0·016 |
| 17 | 2 | 106 | 107 | 37·6 | 37·3 | Blood examined found normal | 10·6 | 0·192 | 42 | 0·181 | 75·5 | 7·6 | 1·02 | 1·20 | 0·008 | 0·189 | +0·003 |
| 18 | 2 | — | | — | | Urine dark ... ... ... | 12·0 | 0·222 | 47 | 0·194 | — | — | 1·03 | 1·13 | 0·025 | 0·219 | +0·003 |
| 19 | 2 | — | | — | | „ ... ... ... | 8·8 | 0·155 | 35 | 0·178 | — | — | 1·20 | 1·54 | 0·045 | 0·213 | −0·058 |
| 20 | 2 | — | | — | | „ ... ... ... | 10·2 | 0·184 | 41 | 0·178 | — | — | 1·24 | 1·69 | 0·017 | 0·195 | −0·011 |
| 21 | 2 | 104 | 106 | 37·4 | 37·4 | Animals quiet, fur ruffled ... | 11·6 | 0·244 | 46 | 0·162 | — | — | 1·38 | 1·82 | 0·028 | 0·190 | −0·054 |
| 22 | 2 | — | | — | | — | 9·2 | 0·163 | 37 | 0·156 | — | — | — | — | 0·014 | 0·170 | −0·007 |
| 23 | 2 | — | | — | | Animals thin and cold ... | 8·8 | | | | | | | | | | |
| 24 | 2 | 103 | 102 | 37·2 | 37·0 | Urine scanty ... ... ... | 6·4 | 0·290 | 61 | 0·320 | 72·7 | 7·5 | 0·74 | 1·11 | 0·043 | 0·363 | −0·070 |
| 25 | 2 | 100 | 99 | — | | „ ... ... ... | 7·3 | | | | | | | | | | |
| 26 | 2 | — | | — | | „ ... ... ... | 7·3 | 0·282 | 58·5 | 0·308 | — | — | — | — | 0·043 | 0·351 | −0·069 |
| 27 | 2 | — | | — | | Animals unwell ... ... | 10·0 | | | | | | | | | | |
| 28 | 2 | 100 | 98 | 37·0 | 36·8 | „ „ ... ... | 15·0 | 0·499 | 100 | 0·384 | — | — | — | — | 0·034 | 0·418 | +0·071 |
| 29 | 2 | — | | — | | „ „ ... ... | | | | | | | | | | | |
| 30 | 2 | 97 | 96 | — | | „ „ ... ... | 19·0 | 0·389 | 76·2 | 0·405 | 79·0 | 7·3 | 0·66 | 0·85 | 0·058 | 0·463 | −0·074 |
| 31 | 2 | — | | — | | Urine scanty ... ... ... | | | | | | | | | | | |
| 32 | 2 | 97 | 92 | — | | Animals thin and cold ... | 31·5 | 0·662 | 126 | 0·652 | — | — | — | — | 0·051 | 0·703 | −0·041 |
| 33 | 2 | — | | — | | Blood examination normal ... | | | | | | | | | | | |
| 34 | 2 | 94 | 92 | 36·2 | 35·9 | Rat 1 killed for examination | 18·0 | 0·380 | 72·2 | 0·362 | 79·5 | 8·0 | 0·92 | 1·17 | 0·050 | 0·410 | −0·030 |
| 35 | 1 | 96 | | 36·0 | | Devoured new diet ravenously | 13·5 | 0·326 | 48·7 | 0·176 | — | — | — | — | 0·030 | 0·106 | +0·220 |
| 36 | 1 | 106 | | — | | Rat much firmer and warmer | 14·0 | 0·340 | 50·5 | 0·097 | — | — | 0·72 | 0·79 | 0·027 | 0·124 | +0·216 |
| 37 | 1 | 111 | | 37·8 | | — | 15·5 | 0·376 | 56·0 | 0·173 | — | — | — | — | 0·024 | 0·197 | +0·179 |
| 38 | 1 | 124 | | — | | Rat very well ... ... | 14·0 | 0·340 | 50·5 | 0·101 | — | — | — | — | 0·022 | 0·123 | +0·217 |
| 39 | 1 | 130 | | 38·2 | | — | 17·5 | 0·425 | 63·2 | 0·186 | — | — | 0·82 | 0·84 | 0·019 | 0·205 | +0·220 |
| 40 | 1 | 130 | | — | | — | 15·8 | 0·382 | 57·0 | 0·121 | — | — | — | — | 0·029 | 0·150 | +0·232 |
| 41 | 1 | 136 | | 38·2 | | Rat 2 killed for examination | 14·3 | 0·345 | 51·6 | 0·123 | — | — | — | — | 0·026 | 0·149 | +0·196 |

P.M. examination rat 1: The skeletal musculature was markedly wasted. There was slight enlargement of the right heart, with congestion of the kidneys and liver. Thyroid gland appeared to be smaller than normal. Other organs normal.

P.M. examination rat 2: The animal presented a normal appearance. Thyroid gland was of normal size.

during that period. Small traces of creatine are often present in rats' urine upon normal diets [Folin and Morris, 1913].

(*b*) *Body-weight.* The two control animals fed upon the complete ration (Diet 1) showed a normal rate of growth throughout the experiment. Similarly the rats studied in Table II grew at a normal rate during the preliminary and final periods, when receiving that diet. During the period when they received the diet free from the water-soluble factor (Diet 2) there was complete inhibition of growth, and a very gradual decline in body-weight. A very large number of rats have been observed whilst being fed for considerable periods upon dietaries deficient in the water-soluble substance. In the case of young rats the withdrawal of this dietary component is almost invariably followed by an immediate cessation of growth. Such inhibition is usually followed by a period during which the animal either maintains a constant body-weight, or shows a very slow decline. Sooner or later there is a sudden and rapid drop in weight lasting a few days, terminated by death. If, during the periods of maintenance or decline, provided in the latter case that the condition of the rat is not extreme, the missing dietary factor is supplied in an adequate amount, the rat recovers and resumes a normal rate of growth. From a large number of observations it has been ascertained that the period of maintenance, which follows the restriction and precedes the final decline, is roughly proportional to the age of the animal when the restriction is imposed. Some average figures which illustrate this are given in Table III.

TABLE III.

| Average body-weight of rat when water-soluble factor withheld from diet | Number of observations | Average duration in days of period of maintenance preceding final decline |
|---|---|---|
| 50 g. | 42 | 25 |
| 60 „ | 67 | 31 |
| 70 „ | 30 | 37 |
| 100 „ | 21 | 46 |
| 150 „ | 12 | 57 |
| 200 „ | 9 | >60 |

Naturally there are wide variations to be observed in animals of all ages, but the above averages are significant of the very great importance of the water-soluble accessory substance during the earlier stages of growth. Adult animals are able to live for considerable periods without receiving a supply of this dietary factor. During this period they usually show a slow decline in body-weight, which is associated at a later stage with an enfeebled resistance to disease. The ultimate decline is, however, inevitable. From the figures given in Table III it is reasonable to assume that the need of the

## TABLE IV.

*Diet deficient in water-soluble accessory substance given at *. Normal diet given at †.*

| No. | | Days before parturition | | | | | Days after parturition | | | | | | | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 8 | 6 | 4 | 2 | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | |
| 1 | Wt. of Mother | 126 | 129* | 135 | 139 | 110 | 107 | 101 | 102 | 100 | 90 | — | — | — | — | — | — | — | — | Five young born, four devoured 1st day; remaining one 3rd day. Mother died with sepsis. No attempt to rear young. |
| | „ Young | — | — | — | — | 25 | 4·5 | — | — | — | — | — | — | — | — | — | — | — | — | |
| 2 | Wt. of Mother | 137 | 141* | 140 | 137 | 101 | 107 | 110 | 117 | 100 | 107† | 114 | 121 | 135 | — | — | — | — | — | Five young born, three born dead. Remaining two devoured first day. |
| | „ Young | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | |
| 3 | Wt. of Mother | 150 | 159 | 160* | 162 | 115 | 115 | 110 | 110 | 106 | 104 | 104† | 114 | 120 | 124 | 132 | 132 | — | — | Seven born, two killed to reduce size of litter, two died 6th day, one died 8th, 11th and 12th days. |
| | „ Young | — | — | — | — | 25 | 30 | 31 | 30 | 19 | 13 | — | — | — | — | — | — | — | — | |
| 4 | Wt. of Mother | 175* | 180 | 182 | 182 | 132 | 120 | 116 | 106 | — | — | — | — | — | — | — | — | — | — | Litter born during night. All but one devoured. That one dead. Mother died with septic condition of uterus 8th day. |
| | „ Young | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | |
| 5 | Wt. of Mother | — | — | 156 | 160* | 140 | 140 | 135 | 131 | 132 | 127 | 129 | 120 | 120 | 120† | 117 | 132 | 137 | 145 | Two young born. |
| | „ Young | — | — | — | — | 20 | 25 | 32 | 34 | 36 | 38 | 38 | 37 | 34 | 32 | 34 | 40 | 54 | 63 | |
| 6 | Wt. of Mother | — | — | 186 | 189* | 149 | 138 | 141 | 138 | 140 | 133 | 132 | 130 | 126 | 121† | 141 | 144 | 148 | 154 | Five young born. One died 17th day. |
| | „ Young | — | — | — | — | 37 | 43 | 50 | 70 | 82 | 88 | 86 | 82 | 84 | 69 | 65 | 70 | 79 | 86 | |
| 7 | Wt. of Mother | — | — | 175 | 176* | 132 | 128 | 128 | 128 | 129 | 123 | 125 | 120 | 118 | 111† | 125 | 129 | 129 | 135 | Nine young born, three killed to reduce size of litter. |
| | „ Young | — | — | — | — | 36 | 39 | 43 | 49 | 67 | 86 | 94 | 96 | 92 | 90 | 94 | 98 | 101 | 115 | |
| 8 | Wt. of Mother | — | 150 | 163 | 177 | 147 | 140 | 140 | 141* | 140 | 137 | 137 | 135 | 132 | 130 | 132† | 139 | 140 | 142 | Five young born. |
| | „ Young | — | — | — | — | 29 | 34 | 46 | 52 | 56 | 62 | 77 | 72 | 70 | 64 | 62 | 62 | 64 | 70 | |
| 9 | Wt. of Mother | 160 | 168 | 172 | 186 | 149 | 148 | 142 | 140 | 142* | 140 | 137 | 137 | 135 | 134 | 132 | 130 | 131 | 134 | Six young born. Three killed to reduce size of litter. |
| | „ Young | — | — | — | — | 19 | 24 | 35 | 42 | 50 | 62 | 67 | 70 | 74 | 72 | 70 | 67 | 60 | 54 | |

organism for the water-soluble factor during the very early stages of growth must be of an urgent nature. McCollum, Simmonds and Pitz [1916] found that the two dietary accessories, the "fat-soluble A" and the "water-soluble B" are only present in the milk of the nursing mother (rat) when they are supplied to her in her own dietary; it being, therefore, improbable that the animal organism can synthesise either substance. The experimental results of these authors regarding the latter substance have been confirmed by a series of experiments made in this laboratory. A number of healthy, full-grown female rats were selected from the breeding stock, all of which had reared at least one satisfactory litter previously. They were mated with reliable breeding males, and as soon as pregnancy was observed, they were removed to separate nesting cages provided with an ample supply of fine wood-shavings for bedding. At various stages, before and after parturition, individual females were placed upon the ration deficient in the water-soluble factor, and the influence of this deficiency upon the mother and young was closely noted. The results of this experiment are summarised in Table IV. From a consideration of the results contained in that table it will be seen that a restriction in the supply of the water-soluble factor in the diet of a pregnant rat may be followed by very serious consequences, both to herself and her offspring. This was particularly marked when the restriction was imposed before parturition. When imposed at, or after, delivery, the young usually lived, and in some cases even showed a subnormal rate of growth for a short period, but the fatal decline was not long in appearing. By returning the mother to a normal diet, the young could be saved, although the improvement in them was not immediately appreciable.

Further points concerning the relationship of the water-soluble factor to growth, will be considered in the later section which deals with its influence upon food-consumption.

(*c*) *Body-temperature.* The rectal temperature of the rats, whose metabolism was being studied, was taken regularly. It was found that a slow fall in body-temperature occurred in the two animals fed upon the inadequate ration. In order to confirm this observation, the body-temperatures of a large number of rats of various ages upon normal diets, and upon diets deficient in the water-soluble factor, were registered. The readings were taken at the same times during the day, as it was found that the normal rat shows considerable diurnal variation in body temperature. All the rats examined were under adult size (140 gms.), and the following average figures were obtained:

TABLE V.

| Sex | Time | Average normal temperature | Average temperature after 10 days upon inadequate diet | Average temperature after 21 days upon inadequate diet |
|---|---|---|---|---|
| Male | 10 a.m. | 38·1° | 37·8° | 37·0° |
| | 6 p.m. | 38·5° | 37·9° | 37·2° |
| Female | 10 a.m. | 38·4° | 38·0° | 37·1° |
| | 6 p m. | 38·7° | 38·3° | 37·5° |

(*d*) *General Health and Conditions of the Organs.* Apart from the metabolism study reported in these pages, the researches carried out in this laboratory during the last year or two have provided an opportunity for observing the state of health of a large number of rats fed upon diets deficient in the factor being considered in this paper. Whilst many of these observations were merely of a cursory nature, the general appearance of the animal alone being recorded, others were more complete, including observations of body temperature, cellular content of the blood, careful post-mortem examinations, and histological examination of the individual organs.

For some time after the water-soluble factor has been withheld from the diet the general appearance of the animals is apparently normal. They are lively, vigorous, sleek-coated and bright-eyed. As the period of maintenance at constant body-weight draws to a close, the rats gradually lose this appearance. The fur loses its gloss and becomes ruffled, whilst the rats assume a hunched attitude, and become lethargic. During the final decline the rats appear pale and emaciated, feeling cold and thin to the touch, and they usually remain huddled in corners away from the light. Funk and Macallum [1915] have recorded that rats fed upon diets deficient in the water-soluble factor (yeast) showed a condition of spastic contraction resembling avian beri-beri, or infantile tetany, 24 or 48 hours before death. Only three cases showing symptoms at all suggestive of nerve disorders have been noted in this laboratory during the careful observation of a very large number of rats upon such diets. All three cases showed a uniformity of symptoms, usually beginning with an apparent difficulty in co-ordinating the movements of the hind limbs, which appeared about 24 hours before death. The incoordination became progressively worse, until there was partial paresis of the hind quarters. As the fatal termination approached, these animals periodically passed through violent convulsive spasms lasting a minute or two. During such attacks the loss of control over the hind quarters was complete, the head was rolled from side to side, and respiration became spasmodic, being assisted by violent diaphragmatic contractions. An attempt was made to

cure one rat showing these advanced symptoms, by an administration of a yeast extract, but it was unsuccessful, the animal dying an hour later.

A number of blood examinations were made upon rats during the period of maintenance. In practically every case normal values for cell counts and haemoglobin percentage were obtained. It is of interest to note that the cellular counts noted for the rats investigated in this work (black variety of *Mus norvegicus*) were uniformly lower than the average figures given for the albino rat by Taylor [1916]. His observation of the wide variations in the differential counts of normal rats was confirmed. The figures obtained are given in Table VI.

TABLE VI.

| | Young adult rat normal diet; average of 12 observations | Young adult rats deficient diet; average of 14 determinations | Adult albino rat (Taylor) |
|---|---|---|---|
| Red corpuscles | $(8–9·7) \times 10^6$ | $(7·8–9·5) \times 10^6$ | $(9–10·5) \times 10^6$ |
| White „ | 8,000–14,000 | 7,000–12,000 | 12,000–30,000 |
| Polymorphonuclears | 30 | 31 | 50 |
| Large mononuclears | 24 | 32 | 14 |
| Small „ | 30 wide variation | 29 wide variation | 30 wide variation |
| Transitional | 15 | 7 | 5 |
| Eosinophile | 1 | 1 | 1 |

The cause underlying the fatal termination which invariably follows the absence of the water-soluble factor from the diet has not been ascertained. A considerable number of rats which have died from the consequences of this deficiency have been subjected to post-mortem examination. The majority presented an emaciated appearance, the wasting being limited chiefly to the skeletal musculature. Enlargement of the right heart was noted in a few cases, whilst a number of animals showed very small thyroid glands. A normal macroscopic appearance of the lungs, kidneys, pancreas, suprarenals, liver, spleen and alimentary tract was always observed. No examination of the nervous system was made. This may appear regrettable, when the possible relationship between this condition and the so-called avian polyneuritis is considered, but no opportunity for the study being made presented itself. Tissues removed from animals at post-mortem examination were subjected to histological examination. Testicles removed from adult males showed in several cases an inhibition of spermatogenesis. The results of the histological examination will furnish the subject of a separate communication.

Funk and Douglas [1914] examined the organs from a number of pigeons

which had developed symptoms of the so-called polyneuritis, as a result of continued feeding upon a diet of polished rice. They recorded complete atrophy of the thymus, whilst the other glands examined all showed a diminution in size, with microscopic evidence of degenerative change in the cells with higher function. The testes were atrophied and spermatogenesis was absent. The thyroid, although atrophied, did not show abnormalities regarding the colloid content.

That inadequacies in the diet of animals may adversely influence breeding is known to all who have have had experience in feeding artificial rations.

That the inadequacy under consideration affects the male rat in this manner was shown by the following breeding trials. Six adult bucks, all known to be good breeders, were placed upon the diet deficient in the water-soluble substance (Diet 2). On the first day one animal was mated with two good breeding females who were receiving normal food, and was allowed to run with them for 48 hours. During this period the sexes were separated to receive their respective diets every morning and evening, and then allowed to run together again. Every seven days another buck was mated with two normally fed does for the same period, under exactly the same conditions. After having run with the females, each male rat was killed, the testicles being removed for histological examination. The results are given in summarised form below:

TABLE VII.

| No. of male | Days upon inadequate dietary before given access to females | Result of mating |
|---|---|---|
| 1 | 0 | 2 pregnancies, 15 young, all reared |
| 2 | 7 | 1 pregnancy, 3 young, all reared |
| 3 | 14 | no pregnancy |
| 4 | 21 | " |
| 5 | 28 | " |
| 6 | 35 | " |

(*e*) *Food-consumption.* From the figures given in Table II, it may be observed that a marked fall in the food-consumption of the rats occurred when the diet was deficient in the water-soluble factor. This has been repeatedly observed with large numbers of other rats, although it was more apparent in some cases than in others. The significance of the calorific intake in such studies as these has been made the subject of several investigations.

Hopkins [1912] submitted this question to a very complete examination and showed conclusively that growth was inhibited in young rats, by the absence of the accessory substances, at a time when the food intake of the

animals was more than sufficient quantitatively to maintain normal growth. He also found that whilst the absolute food-consumption of animals rapidly growing upon diets containing the accessory substances was greater than of those not growing upon the deficient diet, yet the consumption per unit live weight was, in comparable groups, nearly the same. He therefore concluded that any effect of the accessory substances upon the appetite must be secondary to a more direct effect upon the growth processes. The majority of investigators have observed that the absence of the water-soluble factor causes a lowered food-consumption [Funk and Macallum, 1915; Osborne and Mendel, 1917]. The latter authors have discussed the question in the light of some experiments they made, and concluded that yeast added to a diet deficient in the "water-soluble B" exerts some favourable influence upon the metabolism of the rat, causing it to eat more because its general condition is improved. This is confirmatory of the views expressed by Hopkins [1912].

A number of experiments bearing upon this subject were made during the course of the present investigation.

Two batches of twelve young rats were taken. One lot was fed upon the complete ration (Diet 1), the second batch receiving the inadequate diet (Diet 2). The food-consumption and body-weight of the animals were recorded daily, and were as follows:

TABLE VIII.

| | Inadequate diet (3·95 cals. per g.) | | | Complete diet (3·34 cals. per g.) | | |
|---|---|---|---|---|---|---|
| Day | Average wt. g. | Average food consumption g. per day | Average calorific intake per 100 g. wt. per day | Average wt. g. | Average food consumption g. per day | Average calorific intake per 100 g. wt. per day |
| 1 | 89 | 6·6 | 29·4 | 90 | 13·6 | 50·5 |
| 5 | 91 | 6·3 | 27·5 | 110 | 14·3 | 45·0 |
| 9 | 90 | 6·7 | 29·4 | 123 | 15·6 | 42·4 |
| 13 | 87 | 6·4 | 29·2 | 135 | 16·7 | 40·2 |
| 17 | 86 | 6·0 | 27·6 | 149 | 17·1 | 38·4 |
| 21 | 85 | 5·7 | 26·6 | 156 | 18·0 | 38·6 |

As the calorific intake per 100 g. live weight was very low upon the inadequate diet, it was of importance to ascertain whether growth was possible upon such intake, provided that the accessory factor under consideration was present. The following ration was used:

| | | | |
|---|---|---|---|
| Caseinogen ... | 20 parts | Salt mixture | 5 parts |
| Starch ... | 25 ,, | Yeast extract | 5 ,, |
| Butter-fat ... | 20 ,, | Water ... | 20 ,, |
| Agar ... | 5 ,, | | |

The calorific value was approximately 3·7 calories per g. A series of healthy young rats were selected and weighed quantities of this food were supplied to them daily. The results are tabulated in Table IX.

TABLE IX.

| | 1st day | 8th day | 15th day | 22nd day | 29th day | 36th day | 43rd day |
|---|---|---|---|---|---|---|---|
| 1. Food per day, g.* ... ... ... | 8 | 8 | 8 | 8 | 8 | 20 | 20 |
| Average calories per 100 g. live weight | 35·2 | 27 | 24·3 | 22·5 | 21·8 | 52·8 | 45·8 |
| Average yeast extract per 100 g. weight | 0·48 | 0·37 | 0·33 | 0·30 | 0·29 | 1·77 | 1·54 |
| Weight of rat, g. ... ... ... | 84 | 109 | 122 | 132 | 136 | 140 | 161 |
| 2. Food per day, g. ... ... ... | 8 | 8 | 8 | 8 | 8 | 24 | 24 |
| Average calories per 100 g. live weight | 35·2 | 27 | 24·8 | 22·9 | 22·5 | 63·6 | 55·9 |
| Yeast extract per 100 g. live weight ... | 0·48 | 0·37 | 0·33 | 0·30 | 0·30 | 0·72 | 0·63 |
| Weight of rat, g. ... ... ... | 84 | 110 | 120 | 130 | 132 | 139 | 159 |
| 3. Food per day, g. ... ... ... | 8 | 8 | 8 | 8 | 8 | 20 | 24 |
| Average calories per 100 g. live weight | 36·6 | 28·8 | 26·5 | 26·1 | 24·4 | 62·0 | 62·5 |
| Yeast extract per 100 g. live weight ... | 0·48 | 0·38 | 0·36 | 0·36 | 0·33 | 0·78 | 0·71 |
| Weight of rat, g. ... ... ... | 81 | 103 | 112 | 114 | 122 | 129 | 142 |
| 4. Food per day, g. ... ... ... | 8 | 8 | 4 | 5 | 10 | 10 | 10 |
| Average calories per 100 g. live weight | 37·5 | 31·5 | 16·0 | 19·0 | 36·8 | 33·0 | 28·4 |
| Yeast extract per 100 g. live weight ... | 0·48 | 0·42 | 0·21 | 0·19 | 0·60 | 0·53 | 0·46 |
| Weight of rat, g. ... ... ... | 79 | 95 | 93 | 97 | 100 | 112 | 130 |
| 5. Food per day, g. ... ... ... | 8 | 8 | 4 | 5 | 10 | 10 | 10 |
| Average calories per 100 g. live weight | 33·9 | 26·5 | 13·5 | 17·4 | 33·0 | 29·8 | 26·8 |
| Yeast extract per 100 g. live weight ... | 0·46 | 0·36 | 0·18 | 0·23 | 0·54 | 0·48 | 0·43 |
| Weight of rat, g. ... ... ... | 88 | 112 | 110 | 106 | 112 | 124 | 138 |
| 6. Food per day, g. ... ... ... | 8 | 8 | 4 | 5 | 10 | 10 | 10 |
| Average calories per 100 g. live weight | 32·6 | 24·8 | 13·3 | 17·2 | 33·0 | 30·8 | 28·4 |
| Yeast extract per 100 g. live weight ... | 0·44 | 0·33 | 0·18 | 0·23 | 0·54 | 0·50 | 0·46 |
| Weight of rat, g. ... ... ... | 91 | 120 | 112 | 108 | 112 | 120 | 130 |

* Weight of food recorded was given daily for week following.

The figures given in this table demonstrate that rats may show growth, at a corresponding period of development, upon a calorific intake lower than 29 calories per 100 g. live weight, provided that the accessory substance is supplied in adequate amount. Hopkins has stated [1912], "Only those perhaps who have had experience of feeding animals with excess of food, and have noted the amount eaten for considerable periods, will realise how well adjusted under normal circumstances is the instinctive appetite to the physiological needs."

Many opportunities for observing the truth of this statement have been met with during the course of the present research.

In the absence of the water-soluble accessory factor it is probable that the calorific intake is lowered approximately to that required for maintenance at the stage of development which has been reached. This may

be illustrated by the results obtained in a series of feeding experiments designed to ascertain whether food consumption is affected by the calorific value of the diet, when the water-soluble factor "B" is absent.

The following dietaries were prepared:

| | High calorific diet | Low calorific diet |
|---|---|---|
| Caseinogen ... ... | 20 parts | 20 parts |
| Starch ... ... | 35 „ | 45 „ |
| Butter-fat ... ... | 30 „ | 10 „ |
| Salt mixture ... | 5 „ | 5 „ |
| Water ... ... | 10 „ | 40 „ |
| Calorific value per g. | 5·06 | 3·04 |

TABLE X.

| | Low calorific diet (12 rats) | | | | High calorific diet (12 rats) | | | |
|---|---|---|---|---|---|---|---|---|
| Days | Average body-weight g. | Average food consumed daily g. | Average daily calorific intake | Average calories per 100 g. live weight | Average body-weight g. | Average food consumed daily g. | Average daily calorific intake | Average calories per 100 g. live weight |
| 0 | 78 | 6·9 | 21·0 | | 76 | 4·2 | 21·2 | |
| 4 | 79 | 9·0 | 27·3 | | 72 | 5·7 | 28·9 | |
| 8 | 81 | 8·5 | 25·9 | | 69 | 4·4 | 22·3 | |
| 12 | 76 | 8·4 | 25·6 | | 69 | 5·2 | 26·4 | |
| 16 | 76 | 7·7 | 23·4 | 32·0 | 69 | 4·5 | 22·7 | 34·2 |
| 20 | 74 | 7·6 | 23·1 | | 69 | 5·0 | 25·4 | |
| 24 | 72 | 7·7 | 23·4 | | 74 | 4·3 | 21·8 | |
| 28 | 71 | 8·2 | 24·9 | | 69 | 5·1 | 25·9 | |
| | Animals given (diet 1) 5 % yeast extract diet (3·37 cals. per g.) | | | | Animals given (diet 1) 5 % yeast extract diet (3·37 cals. per g.) | | | |
| 29 | 72 | 13·5 | 45·6 | 63·4 | 70 | 12·0 | 40·5 | 57·9 |
| 33 | 89 | 15·6 | 52·7 | 59·0 | 78 | 12·8 | 43·1 | 55·3 |
| 37 | 101 | 16·0 | 54·0 | 53·4 | 91 | 13·3 | 44·9 | 49·4 |
| | Animals given modified diet 1, 5 % meat extract instead of yeast extract (3·25 cals. per g.) | | | | | | | |
| 38 | 106 | 11·3 | 36·7 | | 109 | 14·6 | 49·2 | 45·0 |
| 42 | 105 | 14·0 | 45·5 | | 121 | 14·8 | 50·0 | 41·3 |
| 46 | 104 | 15·0 | 48·7 | 42·6 | 130 | 16·5 | 55·7 | 42·8 |
| 50 | 105 | 14·6 | 47·5 | | 142 | 17·0 | 57·3 | 40·4 |
| 54 | 105 | 14·2 | 46·1 | | 150 | 18·1 | 61·1 | 40·7 |

The food consumption of two closely similar batches of young rats fed upon these rations was noted, the results being given in Table X. It will be seen that the calorific intakes per 100 g. live weight of both groups approximate closely. The question of the influence of flavouring agents upon the palatibility of the ration and food consumption has received attention from several investigators. Both Hopkins [1912], and Osborne and Mendel [1917], have shown, by administering the accessory substances separately from the main food supply, that the action of these substances is direct upon the

TABLE XI.

| | Group 1 (8 rats) | | | Group 2 (6 rats) | | | Group 3 (6 rats) | | | Group 4 (4 rats) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | Yeast extract g. per day | Average daily food intake, g. | Average weight g. | Yeast extract g. per day | Average daily food intake, g. | Average weight g. | Yeast extract g. per day | Average daily food intake, g. | Average weight g. | Yeast extract g. per day | Average daily food intake, g. | Average weight g. |
| 0 | 0 | 7·2 | 86 | 0·25 | 8·9 | 90 | 0·5 | 10·0 | 80 | 1·0 | 13·2 | 86 |
| 2 | 0 | 8·1 | 89 | 0·25 | 8·9 | 90 | 0·5 | 12·4 | 86 | 1·0 | 14·6 | 89 |
| 4 | 0 | 7·4 | 84 | 0·25 | 10·0 | 92 | 0·5 | 14·1 | 89 | 1·0 | 15·0 | 94 |
| 6 | 0 | 7·6 | 87 | 0·25 | 9·4 | 93 | 0·5 | 14·2 | 90 | 1·0 | 15·7 | 102 |
| 8 | 0 | 7·9 | 87 | 0·25 | 9·7 | 92 | 0·5 | 14·1 | 92 | 1·0 | 17·0 | 105 |
| 10 | 0 | 8·0 | 89 | 0·25 | 9·8 | 94 | 0·5 | 15·0 | 96 | 1·0 | 16·0 | 110 |
| 12 | 0 | 8·2 | 87 | 0·25 | 9·2 | 96 | 0·5 | 13·2 | 99 | 1·0 | 16·7 | 116 |
| 14 | 0 | 8·2 | 85 | 0·25 | 9·7 | 96 | 0·5 | 13·0 | 100 | 1·0 | 17·2 | 122 |
| 16 | 0 | 7·7 | 85 | 0·25 | 9·9 | 95 | 0·5 | 12·7 | 104 | 1·0 | 15·0 | 129 |
| 18 | 0 | 7·4 | 84 | 0·25 | 10·0 | 94 | 0·5 | 13·2 | 110 | 1·0 | 17·9 | 134 |
| 20 | 0 | 7·1 | 86 | 0·25 | 9·7 | 95 | 0·5 | 14·5 | 112 | 1·0 | 19·2 | 137 |
| 22 | 0 | 6·7 | 84 | 0·25 | 11·1 | 97 | 0·25 | 12·0 | 110 | 1·0 | 18·0 | 142 |
| 24 | 0·5 | 10·2 | 86 | 0·75 | 15·0 | 105 | 0·25 | 12·0 | 110 | 1·0 | 20·0 | 149 |
| 26 | 0·5 | 11·1 | 88 | 0·75 | 16·7 | 112 | 0·25 | 13·0 | 109 | 1·0 | 21·2 | 150 |
| 28 | 0·5 | 14·4 | 90 | 0·75 | 17·0 | 119 | 0·25 | 12·0 | 109 | 1·0 | 19·1 | 157 |
| 30 | 0·5 | 13·0 | 93 | 0·75 | 18·7 | 122 | 0·25 | 12·2 | 108 | 0 | 17·2 | 156 |
| 32 | 0·5 | 12·9 | 96 | 0·75 | 18·5 | 130 | 0·25 | 12·2 | 109 | 0 | 15·4 | 150 |
| 34 | 0·5 | 14·2 | 98 | 1·0 | 19·8 | 138 | 0·25 | 11·7 | 107 | 0 | 15·0 | 150 |
| 36 | 0·5 | 15·0 | 110 | 1·0 | 19·4 | 147 | 0·25 | 10·7 | 105 | 0 | 15·6 | 149 |
| 38 | 0·5 | 15·1 | 114 | 1·0 | 19·0 | 150 | 0·25 | 9·8 | 105 | 0 | 14·3 | 147 |
| 40 | 0·5 | 14·6 | 116 | 1·0 | 20·0 | 150 | 0·25 | 9·9 | 104 | 0 | 14·0 | 146 |

growth processes. Commercial meat extract has been found to be deficient in the water-soluble accessory factor [Drummond, 1917]. The influence of this substance, acting as far as is known purely as a flavouring agent, upon food-consumption may be seen from Table X. An increase in calorific intake is apparent without any growth resulting.

Osborne and Mendel [1917] found that the amount of growth is proportional to the amount of yeast fed, the other components of the diet being adequate. Some experiments of this nature were made in this laboratory, mainly with the object of determining the minimum quantity of extract containing the accessory factor which would give growth. Four series of healthy young rats were selected, and placed upon the diet deficient in the water-soluble factor. A standard yeast extract was then prepared, measured quantities of which were administered *per os* to each rat daily by means of a small syringe before the animals received their morning meal. The results are tabulated in Table XI.

From Table XI the following average figures have been extracted (Table XII).

TABLE XII.

| Approximate amount of yeast extract per 100 g. live weight given daily | Result | Animals yielding result |
|---|---|---|
| 0 | Maintenance or slow decline | Groups 1 and 4 |
| 0·25 | Maintenance ... ... | Groups 2 and 3 |
| 0·50 | Slow growth ... ... | Groups 1 and 3 |
| 0·60 | Medium growth ... ... | Groups 1 and 3 |
| 0·70 | Good growth ... ... | Group 2 |
| 1·0—1·5 | Rapid growth ... ... | Group 4 |

These figures show reasonable agreement with some of the other results recorded in these pages. Thus, from Table IX, it will be seen that several rats receiving 8 g. of diet daily, containing 0·4 g. yeast extract, showed cessation of growth upon reaching an average weight of 135 g. that is when ingesting approximately 0·30 g. yeast extract per 100 g. body weight.

Although, as Hopkins has pointed out [1912], it is unwise to expend time upon determinations of this nature until a more or less pure substance has been isolated, it may yet be of interest to note certain facts regarding the composition of the yeast extract. The 0·3 g. contained 0·24 g. of solids, of which approximately 70 % was represented by products known to be inactive as regards growth-accelerating influence, such as inorganic salts. The remaining 0·064 g. contained the accessory factor under consideration, as well as considerable amounts of adenine and leucine.

### 2. *Concerning the presence of the Water-soluble Accessory Substance in actively growing Animal Tissues.*

The water-soluble accessory substance has not been found in association with extracts of animal tissues, such for example as commercial meat extract. Likewise fresh beef tissue has been found during the present research to be deficient in the factor. The question arose as to whether it might be present in tissues in the process of rapid growth. To test this, concentrated protein-free aqueous extracts were prepared from three such tissues, (*a*) rat-embryos, 30 days old; (*b*) rat sarcoma tissue; (*c*) Rous chicken sarcoma tissue. In no case did the administration of these extracts, or the feeding of the fresh tissues themselves correct the inadequacy of the diet deficient in the water-soluble B (Table XIII).

TABLE XIII.

| Nature and percentage of substance added to inadequate diet | Average weight of 6 rats every seven days | | | | | Average period in days of maintenance before final decline |
|---|---|---|---|---|---|---|
| | 0 | 7 | 14 | 21 | 28 | |
| Inadequate ration alone ... ... ... | 64 | 63 | 67 | 63 | 54 | 33 |
| 4 % beef tissue ... ... ... ... | 56 | 59 | 62 | 59 | 49 | 31 |
| 5 % commercial meat extract ... ... | 86 | 87 | 84 | 80 | 80 | 46 |
| 6 % aqueous extract rat embryo ... ... | 58 | 60 | 63 | 56 | 52 | 30 |
| 10 % „ „ rat sarcoma ... | 60 | 66 | 58 | 52 | — | 27 |
| 10 % „ „ fowl sarcoma ... | 56 | 50 | 51 | 51 | 49 | 30 |
| 1 % desiccated pituitary gland ... ... | 47 | 45 | 49 | 50 | 52 | — |
| 5 % desiccated pituitary gland ... ... | 52 | 56 | 50 | 48 | 47 | — |
| 1 % desiccated anterior lobe pituitary ... | 64 | 66 | 70 | 67 | 60 | 46 |
| 3 % desiccated anterior lobe pituitary ... | 74 | 73 | 72 | 71 | 67 | 40 |
| 2 % desiccated thyroid gland ... ... | 60 | 61 | 56 | 52 | — | 24 |
| 5 % desiccated thymus gland ... ... | 77 | 78 | 71 | 70 | 70 | 40 |
| 2 % desiccated testicular tissue ... ... | 46 | 45 | 52 | 47 | 41 | 30 |
| 2 % desiccated ovarian tissue ... ... | 70 | 64 | 60 | — | — | 29 |

### 3. *Relationship of the Water-soluble Substance to the Glands of Internal Secretion.*

More than one author has made the suggestion that the accessory substances exert their influence upon growth by being indispensable for the functionation of one or more of the glands of internal secretion [Hopkins, 1912; Funk, 1914].

During the present investigation several such glands were added in the desiccated form to the diet deficient in the water-soluble accessory and administered to young rats, in order to determine whether any one of them would supply the missing factor. As may be seen from Table XIII, no such result was obtained in any case.

SUMMARY.

1. The nitrogen metabolism of rats fed upon an artificial diet deficient in the water-soluble accessory substance has been studied. The only apparent deviation from the normal noted was the appearance of creatinuria. It was accompanied by a slow wasting of the skeletal muscles.

2. The food consumption of rats fed upon the deficient diet is low, being in all probability reduced to that sufficient to supply the calorific requirements of maintenance.

3. An increased food consumption may be brought about by the addition of flavouring agents such as meat extract to the diet, but unless this agent contains the water-soluble substance no growth results.

4. The addition to the inadequate diet of extracts containing the water-soluble substance causes a greatly increased food intake, immediately followed by growth.

5. The amount of growth is, within certain limits, proportional to the amount of accessory substance added, provided that the diet is adequate in other respects.

6. There is evidence that the length of time that an animal (rat) is able to maintain itself upon a diet deficient in the water-soluble substance without suffering a serious loss of body-weight is directly proportional to the age at which the restriction is imposed.

7. Actively growing animal tissues (embryos, tumours) do not contain appreciable amounts of the "water-soluble B."

8. Desiccated pituitary gland (whole gland and anterior lobe), thyroid, thymus, testicle and ovarian tissues are also deficient in the "water-soluble B."

9. The cause of the fatal decline which inevitably follows a deficiency of the water-soluble substance has not been determined. Symptoms of nerve disorder were noted before death in three cases.

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# III. SUR LA DIGESTION DES PROTÉINES DE LA VIANDE CUITE CHEZ DES CHIENS À CAROTIDES LIÉES.

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DANS un but d'économie, j'ai été amené à utiliser, au cours de recherches sur la digestion de la viande cuite de bœuf, des chiens de 9 à 10 kilogrammes, auxquels, pour d'autres motifs, on avait prélevé à différentes reprises du sang hors de l'une, puis hors de l'autre carotide, en l'espace de trois à quatre semaines. Par suite de ces saignées, on a été obligé de lier plusieurs fois au cou chacune des deux carotides. On a effectué avec la plus stricte aseptie les divers prélèvements de sang et les ligatures des carotides. Chaque saignée a comporté 120 à 150 cc. On a pratiqué la dernière 8 à 10 jours avant l'ingestion de viande. Les plaies du cou nécessitées par les saignées ont parfaitement guéri par première intention sans le moindre symptôme morbide local ou général. Les animaux présentaient un excellent appétit, n'avaient en aucune façon perdu en poids, étaient très gais, et paraissaient en parfaite santé.

Entre la dernière saignée et l'ingestion de viande, on a soumis les chiens au traitement antihelminthique habituel. On les a maintenus 24 à 36 heures à jeûn, tout en leur permettant de boire de l'eau à volonté. Ils ont alors reçu 25 grammes de viande cuite de bœuf par kilogramme de poids. On les a sacrifiés respectivement 3, 7, 8, 9 et 10 heures après ce repas. On a procédé au prélèvement des contenus du corps de l'estomac, de la portion prépylorique de cet organe et de la première partie de l'intestin grêle de la façon décrite dans la communication sur la digestion des protéines de la viande de cuite bœuf, récemment parue dans ce journal [Zunz, 1915]. On a suivi pour la séparation de l'azote coagulable et de l'azote non coagulable, pour la détermination de la répartition de l'azote non coagulable entre les principaux groupes de dérivés des protéines, pour le dosage de l'azote ammoniacal et de

l'azote aminé aliphatique dans chacun de ces contenus, exactement la même technique que dans les recherches effectuées chez des chiens normaux.

Or, à mon grand étonnement, j'ai bientôt constaté que l'évacuation de l'estomac s'effectuait plus lentement chez des chiens à carotides liées que chez des animaux non soumis au préalable à cette intervention opératoire. Il était, par conséquent, interdit de tenir compte lors de l'étude de la digestion normale de la viande cuite de bœuf, des résultats obtenus chez les cinq chiens à carotides liées. J'avais l'intention d'étudier de façon détaillée la digestion de la viande chez des chiens à carotides liées et de rechercher si les saignées répétées exercent ou non une influence sur les processus digestifs, en dehors de celle de la ligature des carotides. Mais, par suite de circonstances indépendantes de ma volonté, il me sera sans doute impossible de m'occuper d'ici à longtemps de cette question. Ceci m'engage à communiquer dès à présent les quelques données expérimentales recueillies sur la digestion des protéines de la viande cuite chez des chiens à carotides liées.

Les Tableaux I à IV résument les constatations effectuées chez des chiens à carotides liées. Ils indiquent 1° la quantité d'azote ingérée; 2° les quantités d'azote existant encore à l'état de protéines intactes dans le corps de l'estomac, dans la région prépylorique de cet organe et dans tout l'estomac; 3° les quantités d'azote trouvées sous forme d'acidalbumine, de protéoses et d'autres dérivés des substances albuminoïdes dans les deux portions de l'estomac, dans l'ensemble de cet organe et dans toute la première partie de l'intestin grêle; 4° les quantités d'azote ammoniacal et d'azote aminé aliphatique renfermées dans les trois portions du tube digestif examinées; 5° les quantités d'azote disparues de l'estomac.

On a calculé les données des Tableaux I à III en % de l'azote ingéré (Tableau V), et en % de l'azote contenu dans la région de l'estomac examinée (Tableau VI), celles des Tableaux I à IV en % de l'azote incoagulable renfermé dans la portion du tube digestif envisagée (Tableau VII).

Même au bout de dix heures, le corps de l'estomac et la portion prépylorique de cet organe renferment des quantités appréciables de bol alimentaire de telle sorte qu'on peut examiner séparément le contenu de chacune des deux portions de cet organe, alors que chez les chiens normaux ceci n'est plus possible dès la septième heure consécutive à l'ingestion de viande cuite de bœuf.

Le nombre des expériences effectuées chez des chiens à carotides liées est beaucoup trop faible pour permettre d'établir des moyennes bien fondées. On doit plutôt se borner à comparer les résultats obtenus au bout des mêmes

## Tableau I.

| Chien en expérience | Poids de l'animal, en grammes | Durée de la digestion, en heures | Quantité de viande ingérée, en grammes | Azote ingéré, en grammes | Azote trouvé dans le corps de l'estomac | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | en grammes | | | | | | | | | en milligrammes | |
| | | | | | à l'état coagulable | à l'état incoagulable | | | | | | | total | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique |
| | | | | | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produits | | | | | |
| | | | | | | | hétéro-albumose et proto-albumose | autres protéoses | total | précipités par l'acide phospho-tungstique | non précipités par l'acide phospho-tungstique | total | | | |
| M | 10200 | 3 | 255 | 8·8206 | 4·6628 | 0·0082 | 0·6459 | 0·2948 | 0·9407 | 0·1589 | 0·1511 | 0·3100 | 1·2589 | 5·9217 | 5·68 | 7·82 |
| N | 9000 | 7 | 225 | 8·1016 | 1·7338 | 0·3306 | 0·2994 | 0·1934 | 0·4928 | 0·2312 | 0·1804 | 0·4116 | 1·2350 | 2·9688 | 21·60 | 37·62 |
| O | 9700 | 8 | 242·5 | 9·4114 | 1·0600 | 0·0188 | 0·2690 | 0·3331 | 0·6021 | 0·4001 | 0·2976 | 0·6977 | 1·3186 | 2·3786 | 49·84 | 75·43 |
| P | 10400 | 9 | 260 | 10·6288 | 1·9740 | 0·0797 | 0·1787 | 0·1437 | 0·3224 | 0·2273 | 0·0485 | 0·2758 | 0·6779 | 2·6519 | 23·31 | 21·86 |
| Q | 8750 | 10 | 219·25 | 8·3052 | 0·7633 | 0·0192 | 0·1023 | 0·3448 | 0·4471 | 0·0437 | 0·0719 | 0·1156 | 0·5819 | 1·3452 | 22·27 | 11·88 |

## Tableau II.

| Chien en expérience | Durée de la digestion, en heures | Azote trouvé dans la région prépylorique de l'estomac | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | en grammes | | | | | | | | | en milligrammes | |
| | | à l'état coagulable | à l'état incoagulable | | | | | | | total | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique |
| | | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produites | | | | | |
| | | | | hétéro-albumose et proto-albumose | autres protéoses | total | précipités par l'acide phospho-tungstique | non précipités par l'acide phospho-tungstique | total | | | |
| M | 3 | 0·0470 | 0·0004 | 0·0218 | 0·0281 | 0·0499 | 0·0150 | 0·0147 | 0·0297 | 0·0800 | 0·1270 | 2·98 | 3·24 |
| N | 7 | 0·1640 | 0·0234 | 0·0412 | 0·0360 | 0·0772 | 0·1016 | 0·0322 | 0·1338 | 0·2344 | 0·3984 | 4·14 | 20·78 |
| O | 8 | 0·1044 | 0·0012 | 0·0073 | 0·0142 | 0·0215 | 0·0364 | 0·0491 | 0·0855 | 0·1082 | 0·2126 | 2·89 | 6·08 |
| P | 9 | 0·1523 | 0·0198 | 0·0452 | 0·0296 | 0·0748 | 0·0520 | 0·0152 | 0·0672 | 0·1618 | 0·3141 | 4·82 | 7·22 |
| Q | 10 | 0·0605 | 0·0008 | 0·0046 | 0·0145 | 0·0191 | 0·0129 | 0·0257 | 0·0386 | 0·0585 | 0·1190 | 3·12 | 4·84 |

## Tableau III.

| Chien en expérience | Durée de la digestion, en heures | Azote trouvé dans l'estomac total | | | | | | | | | | | | Azote disparu de l'estomac, en grammes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | en grammes | | | | | | | | | | en milligrammes | | |
| | | à l'état coagulable | à l'état incoagulable | | | | | | | | | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique | |
| | | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produits | | | total | total | | | |
| | | | | proto-albumose et hétéro-albumose | autres protéoses | total | précipités par l'acide phospho-tungstique | non précipités par l'acide phospho-tungstique | total | | | | | |
| M | 3 | 4·7098 | 0·0086 | 0·6677 | 0·3229 | 0·9906 | 0·1739 | 0·1658 | 0·3397 | 1·3389 | 6·0487 | 8·66 | 11·06 | 2·7719 |
| N | 7 | 1·8978 | 0·3540 | 0·3406 | 0·2294 | 0·5700 | 0·3328 | 0·2126 | 0·5454 | 1·4694 | 3·3672 | 25·74 | 58·40 | 4·7344 |
| O | 8 | 1·1644 | 0·0200 | 0·2763 | 0·3473 | 0·6236 | 0·4365 | 0·3467 | 0·7832 | 1·4268 | 2·5912 | 52·73 | 81·51 | 6·8202 |
| P | 9 | 2·1263 | 0·0995 | 0·2239 | 0·1733 | 0·3972 | 0·2793 | 0·0637 | 0·3430 | 0·8397 | 2·9660 | 28·13 | 29·08 | 7·6628 |
| Q | 10 | 0·8238 | 0·0200 | 0·1069 | 0·3593 | 0·4662 | 0·0566 | 0·0976 | 0·1542 | 0·6404 | 1·4642 | 25·39 | 16·72 | 6·8410 |

## Tableau IV.

| Chien en expérience | Durée de la digestion, en heures | Azote trouvé dans la première portion de l'intestin grêle à l'état incoagulable | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | en grammes | | | | | | | | en milligrammes | |
| | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produits | | | total | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique |
| | | | hétéro-albumose et proto-albumose | autres protéoses | total | précipités par l'acide phospho-tungstique | non précipités par l'acide phospho-tungstique | total | | | |
| M | 3 | 0·0000 | 0·0037 | 0·0260 | 0·0297 | 0·0308 | 0·0476 | 0·0784 | 0·1081 | 6·58 | 7·50 |
| N | 7 | 0·0020 | 0·0012 | 0·0026 | 0·0038 | 0·0279 | 0·0461 | 0·0740 | 0·0798 | 15·75 | 18·71 |
| O | 8 | traces | 0·0019 | 0·0034 | 0·0053 | 0·0478 | 0·0947 | 0·1425 | 0·1478 | 13·38 | 12·79 |
| P | 9 | ,, | 0·0033 | 0·0086 | 0·0119 | 0·0513 | 0·0561 | 0·1074 | 0·1193 | 5·95 | 11·88 |
| Q | 10 | 0·0000 | 0·0046 | 0·0197 | 0·0243 | 0·0618 | 0·0840 | 0·1458 | 0·1701 | 15·47 | 16·90 |

TABLEAU V.

| Chien en expérience | Durée de la digestion, en heures | Portion de l'estomac examinée | Pour-cent de l'azote ingéré retrouvés dans l'estomac | | | | | | | | | | | | disparus de l'estomac |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | à l'état coagulable | à l'état incoagulable | | | | | | | en tout | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique | |
| | | | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produits | | | | | | |
| | | | | | proto-albumose et hétéro-albumose | autres protéoses | total | précipités par l'acide phosphotungstique | non précipités par l'acide phosphotungstique | total | | | | |
| M | 3 | corps ... ... | 52·86 | 0·09 | 7·33 | 3·34 | 10·67 | 1·80 | 1·71 | 3·51 | 14·27 | 67·13 | 0·07 | 0·09 | — |
| | | région prépylorique | 0·53 | 0·01 | 0·24 | 0·32 | 0·56 | 0·17 | 0·17 | 0·34 | 0·91 | 1·44 | 0·03 | 0·04 | — |
| | | estomac total ... | 53·39 | 0·10 | 7·57 | 3·66 | 11·23 | 1·97 | 1·88 | 3·85 | 15·18 | 68·57 | 0·10 | 0·13 | 31·43 |
| N | 7 | corps ... ... | 21·40 | 4·08 | 3·69 | 2·39 | 6·08 | 2·85 | 2·23 | 5·08 | 15·24 | 36·64 | 0·27 | 0·46 | — |
| | | région prépylorique | 2·02 | 0·29 | 0·52 | 0·44 | 0·96 | 1·26 | 0·39 | 1·65 | 2·90 | 4·92 | 0·05 | 0·26 | — |
| | | estomac total ... | 23·42 | 4·37 | 4·21 | 2·83 | 7·04 | 4·11 | 2·62 | 6·73 | 18·14 | 41·56 | 0·32 | 0·72 | 58·44 |
| O | 8 | corps ... ... | 11·26 | 0·20 | 2·86 | 3·54 | 6·40 | 4·25 | 3·16 | 7·41 | 14·01 | 25·27 | 0·53 | 0·80 | — |
| | | région prépylorique | 1·11 | 0·01 | 0·08 | 0·15 | 0·23 | 0·39 | 0·52 | 1·01 | 1·15 | 2·26 | 0·03 | 0·07 | — |
| | | estomac total ... | 12·37 | 0·21 | 2·94 | 3·69 | 6·63 | 4·64 | 3·68 | 8·42 | 15·16 | 27·53 | 0·56 | 0·87 | 72·47 |
| P | 9 | corps ... ... | 18·57 | 0·75 | 1·68 | 1·35 | 3·03 | 2·14 | 0·46 | 2·60 | 6·38 | 24·95 | 0·22 | 0·21 | — |
| | | région prépylorique | 1·44 | 0·18 | 0·43 | 0·28 | 0·71 | 0·49 | 0·14 | 0·63 | 1·52 | 2·96 | 0·04 | 0·06 | — |
| | | estomac total ... | 20·01 | 0·93 | 2·11 | 1·63 | 3·74 | 2·63 | 0·60 | 3·23 | 7·90 | 27·91 | 0·26 | 0·27 | 72·09 |
| Q | 10 | corps ... ... | 9·19 | 0·23 | 1·23 | 4·16 | 5·39 | 0·53 | 0·86 | 1·39 | 7·01 | 16·20 | 0·27 | 0·14 | — |
| | | région prépylorique | 0·73 | 0·01 | 0·06 | 0·17 | 0·23 | 0·15 | 0·31 | 0·46 | 0·70 | 1·43 | 0·04 | 0·07 | — |
| | | estomac total ... | 9·92 | 0·24 | 1·29 | 4·33 | 5·62 | 0·68 | 1·17 | 1·85 | 7·71 | 17·63 | 0·31 | 0·21 | 82·37 |

## Tableau VI.

| Chien en expérience | Durée de la digestion, en heures | Portion de l'estomac examinée | Pour-cent de l'azote contenus dans la région de l'estomac examinée | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | en grammes | | | | | | | | | en milligrammes | |
| | | | à l'état coagulable | à l'état incoagulable | | | | | | | | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique |
| | | | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produits | | | total | | |
| | | | | | hétéro-albumose et proto-albumose | autres protéoses | total | précipités par l'acide phospho-tungstique | non précipités par l'acide phospho-tungstique | total | | | |
| M | 3 | corps ... ... | 78·74 | 0·14 | 10·91 | 4·98 | 15·89 | 2·68 | 2·55 | 5·23 | 21·26 | 0·09 | 0·13 |
| | | région prépylorique | 37·01 | 0·32 | 17·16 | 22·13 | 39·29 | 11·81 | 11·57 | 23·38 | 62·98 | 2·35 | 2·55 |
| | | estomac total ... | 77·86 | 0·14 | 11·04 | 5·36 | 16·40 | 2·86 | 2·74 | 5·60 | 22·14 | 0·14 | 0·18 |
| N | 7 | corps ... ... | 58·40 | 11·13 | 10·09 | 6·51 | 16·60 | 7·79 | 6·08 | 13·87 | 41·60 | 0·73 | 1·27 |
| | | région prépylorique | 41·16 | 5·88 | 10·34 | 9·04 | 19·38 | 25·50 | 8·08 | 33·58 | 58·84 | 1·04 | 5·22 |
| | | estomac total ... | 56·36 | 10·51 | 10·12 | 6·81 | 16·93 | 9·88 | 6·32 | 16·20 | 43·64 | 0·76 | 1·73 |
| O | 8 | corps ... ... | 44·56 | 0·79 | 11·31 | 14·00 | 25·31 | 16·83 | 12·51 | 29·34 | 55·44 | 2·09 | 3·17 |
| | | région prépylorique | 49·11 | 0·56 | 3·43 | 6·63 | 10·11 | 17·12 | 23·09 | 40·21 | 50·89 | 1·36 | 2·86 |
| | | estomac total ... | 44·94 | 0·77 | 10·66 | 13·41 | 24·07 | 16·84 | 13·38 | 30·22 | 55·06 | 2·03 | 3·11 |
| P | 9 | corps ... ... | 74·44 | 3·01 | 6·73 | 5·42 | 12·15 | 8·57 | 1·83 | 10·40 | 25·56 | 0·88 | 0·82 |
| | | région prépylorique | 48·49 | 6·30 | 14·39 | 9·42 | 16·56 | 23·81 | 4·84 | 21·40 | 51·51 | 1·53 | 2·30 |
| | | estomac total ... | 71·68 | 3·36 | 7·55 | 5·84 | 13·39 | 9·42 | 2·15 | 11·57 | 28·31 | 0·95 | 0·98 |
| Q | 10 | corps ... ... | 56·74 | 1·43 | 7·60 | 25·64 | 32·24 | 3·25 | 5·34 | 8·59 | 42·26 | 1·66 | 0·88 |
| | | région prépylorique | 50·84 | 0·67 | 3·87 | 12·18 | 16·05 | 10·84 | 21·60 | 32·44 | 49·16 | 2·62 | 4·07 |
| | | estomac total ... | 56·26 | 1·37 | 7·30 | 24·54 | 31·84 | 3·86 | 6·67 | 10·53 | 43·74 | 1·73 | 1·14 |

## Tableau VII.

| Chien en expérience | Durée de la digestion, en heures | Portion du tube digestif examinée | Pour-cent de l'azote incoagulable contenus dans la région du tube digestif examinée | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | sous forme d'acid-albumine | sous forme de protéoses | | | sous forme d'autres produits | | | à l'état d'azote ammoniacal | à l'état d'azote aminé aliphatique |
| | | | | hétéroalbumose et protoalbumose | autres protéoses | total | précipités par l'acide phosphotungstique | non précipités par l'acide phosphotungstique | total | | |
| M | 3 | corps de l'estomac | 0·65 | 51·32 | 23·40 | 74·72 | 12·63 | 12·00 | 24·63 | 0·46 | 0·62 |
| | | région prépylorique | 0·50 | 27·25 | 35·13 | 62·38 | 18·75 | 18·37 | 37·12 | 3·83 | 4·50 |
| | | estomac total ... | 0·64 | 49·88 | 24·11 | 73·99 | 12·99 | 12·38 | 25·37 | 0·65 | 0·83 |
| | | intestin grêle ... | 0·00 | 3·42 | 24·05 | 27·47 | 28·49 | 44·04 | 72·52 | 6·09 | 6·94 |
| N | 7 | corps de l'estomac | 26·77 | 24·24 | 15·66 | 39·90 | 18·72 | 14·61 | 33·33 | 1·75 | 3·05 |
| | | région prépylorique | 9·98 | 17·58 | 15·36 | 32·94 | 43·34 | 13·74 | 57·08 | 1·77 | 8·87 |
| | | estomac total ... | 24·09 | 23·18 | 15·61 | 38·79 | 22·65 | 14·47 | 37·12 | 1·75 | 3·97 |
| | | intestin grêle ... | 2·51 | 1·50 | 3·26 | 4·76 | 34·96 | 57·77 | 92·73 | 19·74 | 23·45 |
| O | 8 | corps de l'estomac | 1·42 | 20·40 | 25·27 | 45·67 | 30·34 | 22·57 | 52·91 | 3·78 | 5·73 |
| | | région prépylorique | 1·11 | 6·75 | 13·12 | 19·87 | 33·64 | 45·38 | 79·02 | 2·67 | 5·62 |
| | | estomac total ... | 1·40 | 19·37 | 24·34 | 43·71 | 30·59 | 24·30 | 54·89 | 3·70 | 5·71 |
| | | intestin grêle ... | 0·00 | 1·29 | 2·30 | 3·59 | 32·34 | 64·07 | 96·41 | 9·05 | 8·65 |
| P | 9 | corps de l'estomac | 11·76 | 26·36 | 21·20 | 47·56 | 33·53 | 7·15 | 40·68 | 3·44 | 3·22 |
| | | région prépylorique | 12·24 | 27·94 | 18·29 | 46·23 | 32·14 | 9·39 | 41·53 | 2·92 | 4·46 |
| | | estomac total ... | 11·85 | 26·66 | 20·64 | 47·30 | 33·26 | 7·59 | 40·85 | 3·35 | 3·46 |
| | | intestin grêle ... | 0·00 | 2·77 | 7·21 | 9·98 | 43·00 | 47·02 | 90·02 | 5·99 | 9·96 |
| Q | 10 | corps de l'estomac | 3·30 | 17·58 | 59·25 | 76·83 | 7·51 | 12·36 | 19·87 | 3·83 | 2·04 |
| | | région prépylorique | 1·37 | 7·86 | 24·79 | 32·65 | 22·05 | 43·93 | 65·98 | 5·33 | 8·27 |
| | | estomac total ... | 3·12 | 16·70 | 56·10 | 72·80 | 8·84 | 15·24 | 24·08 | 3·97 | 2·61 |
| | | intestin grêle ... | 0·00 | 2·70 | 11·58 | 14·28 | 36·33 | 49·38 | 85·72 | 8·51 | 9·94 |

laps de temps chez les chiens normaux et chez les chiens à carotides liées. Le Tableau VIII permet d'établir cette comparaison pour ce qui concerne l'azote ingéré, existant soit à l'état coagulable, soit à l'état incoagulable dans l'estomac et pour la rapidité de l'évacuation de cet organe. Quant à l'examen séparé des deux portions de l'estomac, la comparaison n'est naturellement possible que pour les chiens sacrifiés 3 et 7 heures après le repas.

Au bout de ces laps de temps, on trouve, chez les chiens à carotides liées, une quantité beaucoup plus considérable d'azote coagulable et d'azote incoagulable dans le contenu du corps de l'estomac que chez les chiens normaux. Il n'y a pas de différences bien nettes sous ce rapport entre les deux catégories d'animaux pour le contenu de la portion prépylorique de l'estomac. Si l'on examine l'ensemble de cet organe, on observe des quantités bien plus notables d'azote à l'état de protéines intactes ou de leurs dérivés chez les chiens à carotides liées que chez les autres. Cette différence entre les deux catégories d'animaux est bien plus marquée pour l'azote coagulable 7 à 10 heures après l'ingestion de viande cuite de bœuf que 3 heures après ce repas.

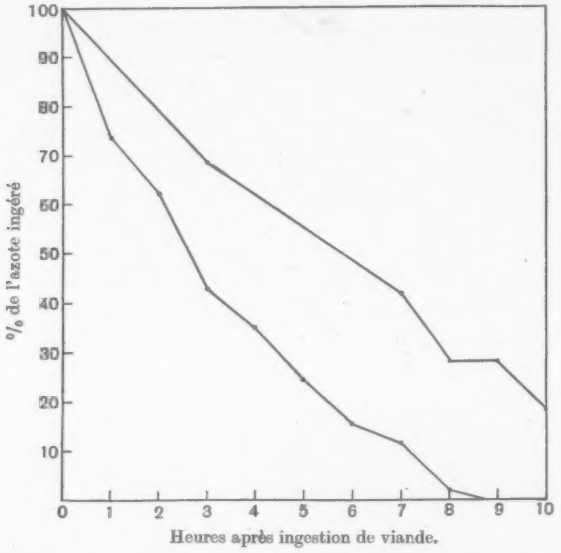
L'azote coagulable l'emporte sur l'azote incoagulable dans le contenu du corps de l'estomac et dans celui de cet organe tout entier, sauf chez le chien O, sacrifié 8 heures après l'ingestion de viande cuite de bœuf. Dans le contenu de la portion prépylorique de l'estomac, l'azote incoagulable l'emporte sur l'azote coagulable chez les chiens M et N, sacrifiés 3 et 7 heures après le repas; les quantités d'azote coagulable et d'azote incoagulable sont par contre à peu près égales chez les trois autres animaux à carotides liées.

Chez les chiens normaux, l'azote coagulable existe toujours en quantité moindre que l'azote incoagulable à partir de la sixième heure consécutive à l'ingestion de viande cuite de bœuf dans le contenu du corps de l'estomac et dans celui de cet organe considéré dans sa totalité. Quant au contenu de la portion prépylorique de l'estomac, il renferme davantage d'azote coagulable que d'azote incoagulable, sauf chez le chien F, sacrifié 6 heures après le repas.

Le Tableau VIII et le tracé ci-contre montrent nettement que l'évacuation de l'estomac s'effectue plus lentement chez les chiens à carotides liées que chez les autres. Au bout de dix heures, on est arrivé chez les premiers environ au même stade qu'au bout de six chez les seconds.

Si l'on se base sur les chiffres d'azote trouvés à l'état coagulable et incoagulable dans l'estomac et sur les proportions d'azote ingéré disparues de cet organe, il semble que les processus digestifs atteignent leur plus grande intensité et que l'évacuation de l'estomac s'effectue le plus rapidement non pas pendant les premières heures consécutives au repas comme chez les

chiens normaux, mais plutôt à une époque se rapprochant dans une certaine mesure de la septième à la huitième heure consécutive à l'ingestion de viande cuite de bœuf. Bien entendu, il ne faut pas perdre de vue qu'on ne dispose jusqu'à présent que de quelques données expérimentales à propos des chiens à carotides liées. Il ne s'agit pour le moment que de présomptions, que des recherches ultérieures effectuées chez de tels animaux, sacrifiés 1, 2, 4, 5, 6, 11 heures et davantage après l'ingestion de viande cuite de bœuf, devront confirmer ou non. On ne doit pas non plus oublier qu'il n'y a pas moyen de tenir compte de l'azote déversé avec les sucs digestifs dans l'estomac et de l'azote résorbé par cet organe, de telle sorte qu'on ne dispose pas, en réalité, de chiffres tout à fait précis, relatifs soit aux quantités d'azote ingéré existant à un moment donné à l'état coagulable et incoagulable dans ce viscère, soit même à la proportion d'azote ingéré ayant disparue alors de l'estomac.




La proportion d'azote incoagulable à l'état d'acidalbumine a été relativement considérable dans le contenu du corps de l'estomac de deux des cinq chiens à carotides liées (chiens N et P); il est vrai qu'un chien normal a présenté un chiffre presque aussi élevé (chien F, sacrifié au bout de 6 heures). Le contenu de la région prépylorique de l'estomac a aussi montré une forte teneur en acidalbumine deux fois sur cinq (chiens N et P). La proportion d'azote incoagulable à l'état d'acidalbumine a même été, chez le chien P,

plus grande dans la portion prépylorique de l'estomac que dans le corps de cet organe. Chez les autres chiens à carotides liées, la teneur en acidalbumine a au contraire diminué du corps de l'estomac à la région prépylorique de cet organe. L'acidalbumine a entièrement fait défaut deux fois sur cinq dans le contenu de la première portion de l'intestin grêle; deux fois on n'en a constaté que des traces. Chez le chien N, chez lequel le contenu du corps de l'estomac et celui de la portion prépylorique de cet organe ont présenté des teneurs relativement considérables en acidalbumine, on a observé dans le contenu de la première partie de l'intestin grêle une teneur en acidalbumine encore appréciable, bien que notablement inférieure à celle de la portion prépylorique de l'estomac.

TABLEAU VIII.

| Laps de temps écoulé depuis l'ingestion de viande, en heures | Portion de l'estomac examinée | Etat des chiens | Pour-cent de l'azote ingéré | | |
|---|---|---|---|---|---|
| | | | existants dans l'estomac à l'état | | disparus de l'estomac |
| | | | coagulable | non-coagulable | |
| 3 | corps ... | normaux ... | 45·61 | 2·81 | — |
| | | à carotides liées | 52·86 | 14·27 | — |
| | portion prépylorique | normaux ... | 1·48 | 1·38 | — |
| | | à carotides liées | 0·53 | 0·91 | — |
| | estomac total | normaux ... | 47·09 | 4·19 | 48·77 |
| | | à carotides liées | 53·39 | 15·18 | 31·43 |
| 7 | corps ... | normaux ... | 1·70 | 2·84 | — |
| | | à carotides liées | 21·40 | 15·24 | — |
| | portion prépylorique | normaux ... | 1·37 | 0·96 | — |
| | | à carotides liées | 2·02 | 2·90 | — |
| | estomac total | normaux ... | 3·07 | 3·80 | 93·13 |
| | | à carotides liées | 23·42 | 18·14 | 58·44 |
| 8 | estomac total | normaux ... | 1·08 | 4·98 | 93·94 |
| | | à carotides liées | 12·37 | 15·16 | 72·47 |
| 9 | estomac total | normaux ... | 0·20 | 3·65 | 96·15 |
| | | à carotides liées | 20·01 | 7·90 | 72·09 |
| 10 | estomac total | normaux ... | 0·45 | 2·23 | 97·32 |
| | | à carotides liées | 9·92 | 7·71 | 82·37 |

Les protéoses représentent, dans le contenu du corps de l'estomac, les 3/4 environ de l'azote incoagulable chez les chiens M et Q, moins de la moitié chez les trois autres animaux à carotides liées, alors qu'on trouve toujours les 3/5 et dans la moitié des cas plus des 4/5 de l'azote incoagulable à cet état chez les chiens normaux. Dans le contenu de la portion prépylorique de l'estomac, moins de la moitié de l'azote incoagulable existe sous forme de protéoses 4 fois sur 5, c'est à dire dans 80 % des cas, chez les animaux à

carotides liées, mais seulement 2 fois sur 7, c'est-à-dire dans 28, 57 % des cas, chez les chiens normaux. Les albumoses représentent moins de 10 % de l'azote incoagulable dans le contenu de la première portion de l'intestin grêle 3 fois sur 5, c'est-à-dire dans 60 % des cas, chez les chiens à carotides liées et par contre 3 fois sur 10, soit dans 30 % des cas, chez les autres. La proportion d'azote incoagulable à l'état de protéoses diminue du contenu du corps de l'estomac à celui de la région prépylorique de cet organe; cette diminution est parfois très accusée (chiens O et Q), parfois seulement très faible (chien P). Elle est toujours fort intense du contenu de la portion prépylorique de l'estomac à celui de la première partie de l'intestin grêle.

Les protéines précipitées par demi-saturation au moyen du sulfate de zinc l'emportent sur les autres: dans le contenu du corps de l'estomac, 3 fois sur 5, soit dans 20 % des cas, chez les chiens à carotides liées, au lieu de 3 fois sur 7 chez les chiens normaux; dans le contenu de la portion prépylorique de l'estomac, 2 fois sur 5, soit dans 40 % des cas, chez les chiens à carotides liées, au lieu de 1 fois sur 7, soit dans 14·28 % des cas, chez les chiens normaux; dans le contenu de tout l'estomac 3 fois sur 5, soit dans 60 % des cas, chez les chiens à carotides liées, au lieu de 3 fois sur 10, soit dans 30 % des cas, chez les chiens normaux.

La proportion d'azote incoagulable à l'état d'hétéro-albumose et de protoalbumose diminue du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci à celui de la première partie de l'intestin grêle, sauf chez le chien P, sacrifié au bout de 9 heures, chez lequel on rencontre une proportion un peu plus élevée de ces produits dans la région prépylorique de l'estomac que dans le corps de cet organe.

La proportion d'azote incoagulable à l'état de protéoses non précipitées par demi-saturation au moyen du sulfate de zinc diminue aussi au fur et à mesure qu'on progresse dans le tube digestif. Toutefois, chez le chien M, il y a davantage d'azote sous cette forme dans la région prépylorique de l'estomac que dans le corps de cet organe. De plus, chez ce même animal, le contenu de la première partie de l'intestin grêle offre une proportion notablement moindre de ces composés que le contenu de la région prépylorique de l'estomac, mais dépassant néanmoins celle du contenu du corps de l'estomac.

On rencontre dans le contenu du corps de l'estomac des chiens à carotides liées 20 à 50 % de l'azote incoagulable à l'état de peptones et de polypeptides, alors que ces substances n'atteignent jamais 20 % de cet azote chez les animaux normaux nourris de la même façon. Dans le contenu de la région prépylorique de l'estomac, on trouve, tant chez les animaux à carotides liées

que chez les autres, 35 à 80 % de l'azote incoagulable sous cette forme. Néanmoins chez le chien normal F, sacrifié 6 heures après l'ingestion de viande cuite de bœuf, les peptones et les polypeptides ne représentent pas le quart de l'azote incoagulable. Dans le contenu total de l'estomac, la proportion d'azote incoagulable à l'état de composés autres que l'acidalbumine et les protéoses varie entre 25 et 55 % chez les chiens à carotides liées. Chez les animaux normaux, l'estomac considéré dans son ensemble ne contient pas dans la majorité des cas (6 fois sur 10) le 1/5 de l'azote incoagulable sous forme de peptones et de polypeptides; ces produits correspondent 2 fois (chiens E et J) environ au quart et deux fois (chiens C et I) à peu près au tiers de l'azote incoagulable.

Quant à la première partie de l'intestin grêle, les composés autre que l'acidalbumine et les protéoses, c'est-à-dire les peptones, les peptides et les acides aminés dépassent toujours 70 % et souvent même 90 % de l'azote incoagulable tant chez les chiens à carotides liées que chez les autres. Chez le chien G, non opéré et sacrifié 7 heures après l'ingestion de viande cuite de bœuf, on n'observe toutefois que 68 % de l'azote incoagulable à cet état dans le contenu intestinal.

La proportion d'azote incoagulable à l'état de dérivés des protéines autres que l'acidalbumine et les protéoses s'accroît, chez les chiens à carotides liées comme chez les animaux normaux, du conten ı du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci au contenu de la première partie de l'intestin grêle. L'accroissement est, il est vrai, insignifiant du corps de l'estomac à la région prépylorique chez le chien P, à carotides liées, sacrifié 9 heures après l'ingestion de viande cuite de bœuf.

Parmi les dérivés des protéines autres que l'acidalbumine et les protéoses, ceux précipités par l'acide phosphotungstique l'emportent sur ceux non précipités par ce réactif: dans le contenu du corps de l'estomac, 4 fois sur 5, soit dans 80 % des cas, chez les chiens à carotides liées, au lieu de 3 fois sur 7, soit dans 42·86 % des cas, chez les chiens normaux; dans le contenu de la portion prépylorique de l'estomac, 3 fois sur 5, soit dans 60 % des cas, chez les chiens à carotides liées, au lieu de 2 fois sur 7, soit dans 28·57 % des cas, chez les chiens normaux; dans le contenu de tout l'estomac, 4 fois sur 5, soit dans 80 % des cas, chez les chiens à carotides liées, au lieu de 4 fois sur 10, soit dans 40 % des cas, chez les chiens normaux; jamais dans le contenu de la première partie de l'intestin grêle chez les chiens à carotides liées, au lieu de 3 fois sur 10, soit dans 30 % des cas, chez les chiens normaux. La réaction du biuret a toujours été positive dans le contenu des trois régions du tube

digestif examinées. Les peptones vraies de Kühne n'ont donc jamais fait défaut dans l'estomac et dans la première partie de l'intestin grêle des 5 chiens à carotides liées, sacrifiés respectivement 3, 7, 8, 9 et 10 heures après l'ingestion de viande cuite de bœuf.

Chez les chiens M et Q, sacrifiés 3 et 10 heures après l'ingestion de viande cuite de bœuf, la proportion d'azote incoagulable à l'état de peptones et d'autres composés précipités par l'acide phosphotungstique mais pas par le sulfate de zinc (polypeptides pour l'estomac, polypeptides et acides aminés pour l'intestin grêle) s'accroît du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci au contenu de la première partie de l'intestin grêle. Chez les chiens N et O, sacrifiés respectivement 7 et 8 heures après le repas, la portion prépylorique de l'estomac renferme une plus forte proportion d'azote incoagulable sous forme de ces produits que l'intestin. Chez le chien O, les trois régions du tube digestif examinées offrent, du reste, à peu près la même proportion d'azote incoagulable à l'état de composés précipités par l'acide phosphotungstique mais pas par le sulfate de zinc. Chez le chien P enfin, sacrifié au bout de 9 heures, la région prépylorique de l'estomac renferme un peu moins d'azote incoagulable sous cette forme que le corps de cet organe et beaucoup moins que la première partie de l'intestin grêle.

Quant à la teneur en composés non précipités par l'acide phosphotungstique, elle s'accroît au fur et à mesure qu'on progresse dans le tube digestif. Il faut toutefois remarquer qu'elle est un peu plus faible, chez le chien N, dans la portion prépylorique de l'estomac que dans le corps de cet organe.

Le Tableau IX facilite la comparaison de la répartition moyenne de l'azote incoagulable entre l'acidalbumine, les protéoses et les autres dérivés des protéines dans les diverses régions du tube digestif chez les chiens à carotides liées et chez les animaux normaux après l'ingestion de viande cuite de bœuf.

Il résulte nettement des chiffres relatés dans le Tableau IX qu'on constate en moyenne davantage d'azote incoagulable sous forme d'acidalbumine d'une part, de dérivés non précipités par le sulfate de zinc d'autre part, moins par contre sous forme de protéoses, tant précipitées que non précipitées par demi-saturation au moyen du sulfate de zinc, dans les diverses portions examinées du canal gastrointestinal chez les chiens à carotides liées que chez les autres.

Les composés précipités par l'acide phosphotungstique mais pas par le sulfate de zinc représentent dans chacune des deux portions de l'estomac en

## Tableau IX.

| Portion du tube digestif examinée | Etat des chiens | Pour-cent de l'azote incoagulable en moyenne sous forme | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | d'acid-albumine | de protéoses | | | d'autres produits | | |
| | | | hétéroalbumose et protoalbumose | autres protéoses | total | précipités par l'acide phosphotungstique | non précipités par l'acide phosphotungstique | total |
| corps de l'estomac... | normaux ... | 4·33 | 42·14 | 41·32 | 83·46 | 6·81 | 5·40 | 12·21 |
| | à carotides liées | 8·78 | 27·98 | 28·96 | 56·94 | 20·54 | 13·74 | 34·28 |
| portion prépylorique de l'estomac | normaux ... | 3·73 | 19·72 | 30·87 | 50·59 | 18·27 | 27·41 | 45·68 |
| | à carotides liées | 5·04 | 17·47 | 21·34 | 38·81 | 29·99 | 26·15 | 56·15 |
| estomac total ... | normaux ... | 4·10 | 34·17 | 41·01 | 75·18 | 10·29 | 10·43 | 20·72 |
| | à carotides liées | 8·22 | 27·16 | 28·16 | 55·32 | 21·67 | 14·79 | 36·46 |
| première portion de l'intestin grêle | normaux ... | 0·03 | 6·76 | 11·25 | 18·01 | 36·94 | 45·02 | 81·96 |
| | à carotides liées | 0·50 | 2·34 | 9·68 | 12·02 | 35·02 | 52·46 | 87·48 |

## Tableau X.

| Portion du tube digestif examinée | Etat des chiens | Azote ammoniacal, en pour-cent | | | | Azote aminé aliphatique, en pour-cent | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | de l'azote incoagulable | | de l'azote total | | de l'azote incoagulable | | de l'azote total | |
| | | chiffres extrêmes | moyenne | chiffres extrêmes | moyenne | chiffres extrêmes | moyenne | chiffres extrêmes | moyenne |
| corps de l'estomac... | normaux ... | 0·03 à 3·25 | 1·40 | 0·01 à 2·03 | 0·58 | 1·25 à 7·09 | 3·93 | 0·15 à 2·51 | 1·27 |
| | à carotides liées | 0·46 à 3·83 | 2·65 | 0·09 à 2·09 | 0·89 | 0·62 à 5·73 | 2·93 | 0·13 à 3·17 | 1·26 |
| portion prépylorique de l'estomac | normaux ... | 0·15 à 5·89 | 3·25 | 0·06 à 3·24 | 1·55 | 3·13 à 13·65 | 7·38 | 1·50 à 3·82 | 2·57 |
| | à carotides liées | 1·77 à 5·33 | 3·30 | 1·04 à 2·62 | 1·78 | 4·46 à 8·87 | 6·34 | 2·30 à 5·22 | 3·40 |
| estomac total ... | normaux ... | 0·05 à 4·04 | 2·21 | 0·01 à 3·79 | 1·38 | 1·29 à 7·45 | 3·53 | 0·22 à 2·38 | 1·46 |
| | à carotides liées | 0·65 à 3·97 | 2·68 | 0·14 à 2·03 | 0·92 | 0·83 à 5·71 | 3·32 | 0·18 à 3·11 | 1·43 |
| première portion de l'intestin grêle | normaux ... | 3·21 à 8·68 | 6·17 | — | — | 5·02 à 28·82 | 16·60 | — | — |
| | à carotides liées | 5·99 à 9·74 | 9·88 | — | — | 6·94 à 23·45 | 11·77 | — | — |

moyenne une proportion plus considérable de l'azote incoagulable chez les chiens à carotides liées que chez les autres, tandis qu'au contraire dans l'intestin grêle, il y a une proportion légèrement moindre de ces composés chez les animaux opérés que chez les non opérés. Quant aux produits non précipités par l'acide phosphotungstique, ils comprennent une proportion plus notable de l'azote incoagulable chez les chiens à carotides liées que chez les autres dans le contenu du corps de l'estomac, dans le contenu total de cet organe et dans le contenu de la première partie de l'intestin grêle, un proportion un peu moindre au contraire chez les premiers que chez les seconds dans la région prépylorique de l'estomac.

Arrivons-en à l'azote ammoniacal et à l'azote aminé aliphatique. La teneur en chacune de ces deux espèces d'azote s'accroît, chez les chiens à carotides liées comme chez les autres, du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de ce dernier à celui de la première partie de l'intestin grêle. Chez le chien N, sacrifié 7 heures après l'ingestion de viande cuite de bœuf, la teneur en azote ammoniacal est néanmoins à peu près la même dans les deux portions de l'estomac. Chez les chiens O et P, sacrifiés respectivement 8 et 9 heures après ce repas, la teneur en azote ammoniacal est même inférieure dans la région prépylorique de l'estomac à celle constatée dans le corps de cet organe. Chez le chien P, la portion prépylorique de l'estomac a une teneur en azote aminé aliphatique très légèrement inférieure à celle constatée dans le corps de cet organe.

L'azote aminé l'emporte sur l'azote ammoniacal sauf dans le contenu intestinal chez le chien O, dans le contenu du corps de l'estomac chez le chien P, dans le contenu total de l'estomac et dans celui du corps de cet organe chez le chien Q.

Le Tableau X permet de comparer les teneurs en azote ammoniacal et en azote aminé aliphatique des diverses régions étudiées du tube digestif après l'ingestion de viande cuite de bœuf chez les chiens à carotides liées d'une part et chez les animaux non opérés d'autre part.

Si l'on calcule la teneur moyenne en azote ammoniacal par rapport à l'azote incoagulable, on constate qu'elle est plus élevée dans les deux portions de l'estomac et dans la première partie de l'intestin grêle chez les chiens à carotides liées que chez les autres. Tel est encore le cas par rapport à l'azote total pour le contenu du corps de l'estomac et pour celui de la portion prépylorique de cet organe considéré isolément. Par contre, pour le contenu total de l'estomac, on trouve moins d'azote ammoniacal en moyenne chez les animaux opérés que chez les autres. Cette discordance tient aux résultats

des chiens normaux H à J, sacrifiés 8 à 10 heures après l'ingestion de viande cuite de bœuf. Chez ces 3 animaux, on a dû se borner à examiner le contenu total de l'estomac et celui-ci possédait une teneur relativement élevée en azote ammoniacal. Or, comme par suite du ralentissement de l'évacuation de l'estomac chez les chiens à carotides liées, on n'a pu examiner des stades aussi avancés des processus digestifs, une comparaison parfaite est impossible entre les deux catégories d'animaux.

La teneur en azote aminé aliphatique, calculée par rapport à l'azote incoagulable, est moindre dans les trois parties du tube digestif examinées chez les chiens à carotides liées que chez les animaux normaux. Si l'on prend comme base de calcul l'azote total, la teneur en azote aminé est sensiblement la même dans le corps de l'estomac et dans l'ensemble de cet organe chez les deux catégories d'animaux; elle est par contre plus considérable chez les chiens à carotides liées que chez les autres dans la portion prépylorique de l'estomac.

Il ne faut pas pour le moment attacher trop d'importance aux différences signalées entre les chiens à carotides liées et les autres pour ce qui concerne les teneurs en azote ammoniacal et en azote aminé aliphatique des diverses régions du tube digestif, car on n'a pas jusqu'à présent examiné un nombre suffisant d'animaux opérés.

En définitive, ce qui caractérise surtout la digestion de la viande cuite de bœuf chez les chiens à carotides liées, c'est le ralentissement de l'évacuation de l'estomac. Ce ralentissement pourrait fort bien être dû à une moindre sécrétion de suc gastrique dans l'estomac. Cannon [1907] a, en effet, prouvé que l'ouverture et la fermeture du pylore sont placées sous le contrôle du degré d'acidité du contenu de l'antre prépylorique d'une part, du contenu duodénal d'autre part.

Les modifications essentielles constatées dans la répartition de l'azote incoagulable après ingestion de viande cuite de bœuf chez les chiens à carotides liées par rapport aux animaux normaux consistent, ainsi que nous l'avons déjà vu, en une moindre proportion de protéoses et en une proportion plus élevée d'acidalbumine et des autres dérivés des protéines. Ces différences entre chiens normaux et chiens à carotides liées sont beaucoup plus marquées dans le contenu du corps de l'estomac que dans celui de la portion prépylorique de cet organe et surtout que dans celui de la première portion de l'intestin grêle. Ces différences dépendent nécessairement du ralentissement de l'évacuation de l'estomac et des motifs de ce ralentissement: moindre sécrétion de suc gastrique ou sécrétion d'un suc moins acide que normalement,

modifications dans les mouvements de l'estomac. Ce sont là des choses à examiner ultérieurement.

A l'heure actuelle nous ignorons complètement les causes réelles des modifications éventuelles de l'activité chimique et mécanique de l'estomac. Il se peut qu'à la suite de la ligature des deux carotides, la circulation ou l'innervation de l'estomac ou toutes deux subissent de façon directe ou indirecte, peut-être seulement au bout d'un certain laps de temps, des modifications et que celles-ci entraînent à leur tour des variations dans le chimisme de l'estomac ou dans les manifestations motrices de cet organe au cours des processus digestifs. Mais il s'agit là de considérations purement hypothétiques. Il appartient à des recherches futures d'apporter la lumière sur ces points.

D'autres problèmes encore s'imposent à notre attention. Si l'on ne procédait qu'à une seule reprise à la ligature de chacune des deux carotides, observerait-on les mêmes effets qu'après les ligatures répétées de ces deux vaisseaux. Ce n'est pas absolument certain, car il semble bien d'après l'examen des carotides liées à plusieurs reprises qu'elles ont subi des modifications dans leur structure (épaississement, induration, etc.) dans un rayon plus ou moins considérable aux environs de ces ligatures, modifications qui ne semblent pas apparaître de suite et que la simple inspection visuelle ne décèle pas.

On peut aussi se demander si les saignées successives ont exercé une influence. On sait, en effet, qu'une saignée abondante entraîne diverses modifications de la composition du sang: hyperglycémie [Claude Bernard, 1887; v. Mering, 1877; Schenk, 1894; Rose, 1903], hypercholesterinémie [Mauriac, 1912], hyperaminoacidémie [György, 1914; Gorchkoff, Grigorieff et Koutoursky, 1914; György et E. Zunz, 1915].

Mais ces modifications signalées soit peu après la saignée, soit quelques heures après celle-ci, dépendent surtout des processus de régénération destinés à ramener le sang à sa concentration et à sa composition habituelle, dont l'organisme tend toujours à maintenir la constance. Elles ont sans doute cessé leurs effets au bout de 8 à 10 jours, moment où l'on a étudié la digestion de la viande cuite de bœuf chez les chiens à carotides liées. Il est donc très probable que le ralentissement de l'évacuation de l'estomac et les autres particularités observées chez les cinq chiens à carotides liées sont uniquement la conséquence de cette intervention.

RÉSUMÉ.

Après ingestion de viande cuite de bœuf, l'évacuation de l'estomac s'effectue plus lentement chez les chiens à carotides liées que chez les chiens normaux. Ce phénomène et les facteurs dont il dépend, entraînent certaines modifications par rapport à la normale dans la composition du contenu du corps de l'estomac, à un moindre degré dans celle du contenu de la portion prépylorique de cet organe, et à un moindre degré encore dans celle du contenu de la première partie de l'intestin grêle. Ces modifications consistent essentiellement en une moindre proportion d'azote incoagulable à l'état de protéoses et en une plus forte proportion tant sous forme d'acidalbumine que sous celle d'autres dérivés des protéines (peptones et polypeptides pour l'estomac; peptones polypeptides et acides aminés pour l'intestin grêle).

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# IV. THE VISCOSITY OF BLOOD.

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## I. Introductory.

Several attempts have been made to correlate the viscosity of blood with the number of corpuscles present [Denning and Watson, 1906; Josué and Maturier, 1916]. All observers agree that the viscosity rises with increases in the number of corpuscles present and that the effect becomes progressively greater with each such increase above a certain critical value. It has also been noticed that blood from different animals shows different viscosities even when the plasma viscosity and the number of corpuscles are the same in each. These observations suggested that the factor contributed by the corpuscles depended on their total volume rather than on their number [McClendon, 1916, p. 180]. Ferrai [quoted by Denning and Watson, 1906] also, who showed that the addition of carbon dioxide to blood caused a rise in viscosity, attributed the rise to a simultaneous increase in the volume of the corpuscles. Thus a qualitative relationship has been established between the total volume of the corpuscles, the viscosity of plasma and the viscosity of whole blood; this paper is an endeavour to make clear the quantitative relationships.

## II. Methods.

Home-made capillary viscosimeters were used of the pattern illustrated in Fig. 1 *a*. They are blown from tubing of 0·8 mm. diameter for the large size, using 2 cc., and 0·2 mm. diameter for the small form, holding 1 cc. In the large model the bulb *C* holds rather less than 1 cc. The other dimensions are in proportion. The funnel *D* is made from a half-inch test-tube drawn out and fused on, and holds about five cubic centimetres. It is necessary to have it of that size to prevent overflow of bubbles, which may form when the blood is blown out of *C* as described later. Two cc. of blood mixed with a little hirudin is placed in *D* and allowed to run into *C*. The instrument is then placed upright in a thermostat at 40° for ten minutes. The bulb *C* is

then emptied into *D* by blowing slowly down the rubber tube *E* until the whole of the blood is in *D*. The corpuscles are mixed with the plasma by blowing a gentle stream of air through or by stirring with a coiled wire[1]. The bubbles which form are destroyed by touching them with a hot needle and the time of flow from *B* to *A* noted with a stopwatch, the blood in *D* being continually stirred with a coiled wire.

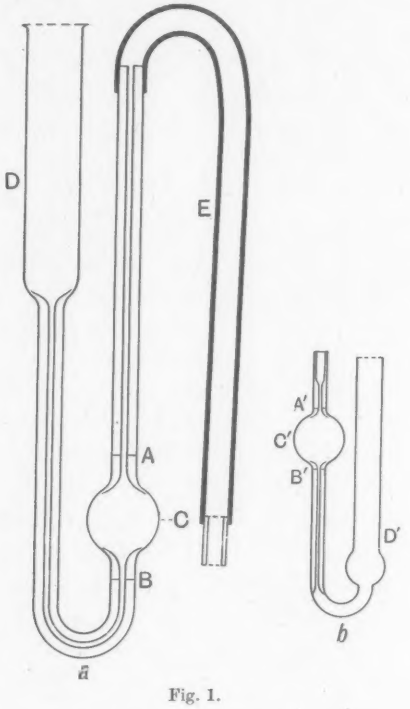


Fig. 1.

## III. Causes of Error.

(*a*) *Conditions setting up turbulent flow.* The transition between the expanded portions and the capillary itself must be a gradual one and especially must not be constricted. One instrument constructed led to much waste of time because of this latter failing.

[1] Blowing air through is of course inadmissible when the effect of small changes in the $CO_2$ content of the blood is in question but all the experiments to be described have been conducted with the blood fully oxygenated, and the changes in viscosity dealt with are very much greater (by as much as 1000 %) than the small changes produced by alterations in the viscosity of the blood.

Not much emphasis is laid on this point in the usual textbook descriptions of viscosity measurements. The magnitude of the error is shown by the following figures (Table I) showing the relation of the time of flow of 7 % gum acacia solution in 2 % sodium chloride (used as having a viscosity similar to that of normal blood) to the time of flow of water at different temperatures and the ratio which gives the "apparent" viscosity.

TABLE I.

A *is a viscosimeter with a constriction,* B *one without.*

| | A | | | B | | |
|---|---|---|---|---|---|---|
| | Time of flow | | | Time of flow | | |
| Temp. | Water | Gum | Ratio | Water | Gum | Ratio |
| 40° | 12·5 | 43·5 | 3·4 | 10 | 43 | 4·3 |
| 16·5° | 19 | 82 | 4·3 | — | — | — |
| 11·5° | — | — | — | 18·0 | 7·9 | 4·45 |

On blowing out the constriction the results were concordant. The error was caused by the formation of eddies at the constriction. Turbulent flow was set up and the amount of energy absorbed by the eddies increases with the velocity of flow and is greater the less viscous the fluid [Osborne Reynolds, 1883]. Consequently the time of flow is increased more for the less viscous fluid and especially at the higher temperatures where the velocities of flow are higher. The "apparent" viscosity of very much more viscous fluids such as the gum solution used in the above experiment is therefore less than the real. Viscosimeters of large bore (greater than 0·5 mm.) should always be calibrated by measuring not only the time of flow of water but also that of gum solutions of known viscosities (as measured in a very fine bore viscosimeter) including the whole range of viscosities to be measured. The use of very fine bore viscosimeters for the measurement of the viscosity of blood introduces chances of large errors due to settling of the corpuscles.

(*b*) *The sedimentation of the corpuscles.* Settling of corpuscles during the course of the transpiration has not attracted the attention it deserves. The rate varies enormously in different species and considerably in different individuals of the same species. The differences depend on no single physical factor. One of the most striking features is the slow settling which sometimes takes place in saline. The size of the error can be seen in the following table.

TABLE II.

*Time of flow of sheep's corpuscles in saline; haematocrit volume* 30 %.

| Sedimentation prevented | Sedimentation not prevented |
|---|---|
| Time of flow | Time of flow |
| 33·4, 33·2, 33·8 | 35·8, 35·8 (error ca. 10 %) |

This suspension settled rather rapidly. The error with a larger proportion of corpuscles (longer times of flow) is a much greater one, for the greater the number of corpuscles present the greater the rate of increase of the viscosity with further increase in concentration (see p. 68). Without stirring I have found it impossible to get concordant results with the higher concentrations of corpuscles used. Nor does the fact that the later portions of the suspension to pass through the capillary tube contain a smaller number of corpuscles than the earlier compensate for this effect: for the transference of a volume of corpuscles from a dilute suspension to a stronger one produces a greater alteration of the viscosity of the strong suspension than of the dilute one (see p. 68). This may be crudely illustrated by the fact that it takes longer to empty a room full of people if everybody jostles around the doorway than if each waits his turn.

The form of viscosimeter usually adopted, with the measuring bulb on the pressure side of the viscosimeter ($A'B'$, Fig. 1 *b*) prevents any stirring during the course of the experiments. This led I think to the differences in "apparent" viscosity which Denning found between large tubes and small. I have been unable to obtain these differences working with tubes of 0·8 mm. and 0·2 mm. diameter. The narrower tubes in their experiments produced a long time of outflow which gave greater opportunities for settling and the higher the concentration reached in any experiment the greater the deviation from the real time of flow, the percentage error increasing during the experiment.

(*c*) *Formation of beads of fluid.* On making measurements with the instrument (Fig. 1 *a*) care must be taken that beads of fluid do not collect in the capillary above $A$ (Fig. 1 *a*). These form from the fluid, which wets the sides of the capillary, if $C$ is emptied too rapidly into $D$. A similar bead may form at $B$ and persisting as a bubble on the top of the inflowing blood, may pass into the capillary above $A$ as a bead. These beads of fluid require very large amounts of energy to drive them along capillary tubes, especially if they contain air bubbles (cf. air embolism in the coronary arteries); and when they reach the open end of the tube a bubble forms and breaks several times. Both these processes increase the time of flow very considerably. In the type of viscosimeter, Fig. 1 *b*, these beads forming there at $A'$ are even more troublesome.

(*d*) *Temperature variations* produce alterations in the viscosity of blood and the alterations are not parallel with that of water; all comparisons were therefore made at the same temperature, viz. 40°.

(*e*) *Use of anticoagulants.* In these experiments hirudin was used. A few fine flakes were placed in the bottom of a short test-tube into which 5 cc. of blood were run. The hirudin was without appreciable influence on the viscosity, as was shown by collecting fowl's blood directly from an artery into a clean glass vessel, measuring its viscosity, adding hirudin and measuring the viscosity again. Defibrination is inadmissible because of its effect on the plasma. Oxalate, citrate and fluoride in the solid state are also inadmissible because of the alteration in the volume of the corpuscles by osmotic changes. The following table gives the alterations produced by the addition of 0·08 % NaCl (corresponding to 0·1 % potassium oxalate and 0·35 % sodium citrate) in the corpuscular volume [Hamburger, 1905] and the consequent alteration in viscosity calculated on the assumption that the plasma viscosity remained constant (see formula established later, p. 68).

TABLE III.

| Corpuscular volume | | Viscosity as a multiple of water | | |
|---|---|---|---|---|
| before adding salt | after adding salt 0·08 % | before adding salt | after adding salt | % error |
| 33·3 % | 36·7 % | 5·0 | 4·61 | 9·8 % |
| 48·6 % | 53·1 % | 6·6 | 5·92 | 10·1 % |
| 58·3 % | 64·1 % | 9·06 | 7·85 | 12·4 % |

On this point see also Burton-Opitz [1911]. In employment of potassium oxalate other complications arise. At certain concentrations potassium oxalate produces large increases in the viscosity of the plasma, large enough sometimes to mask the effect mentioned above. Sodium citrate does not appear to produce this effect. The addition of isotonic solutions of sodium citrate to blood produces no osmotic changes, but causes difficulty by diminishing the viscosity of the plasma to an unknown extent. If the volume of the corpuscles is known, the true viscosity can be calculated by one of the formulae given later, but the method is obviously very cumbrous.

(*f*) *Haemolysis* by bacteria and saponin produces very large rises in viscosity. Centrifuged corpuscles on being allowed to stand in the air, become converted into a tarry mass which will not pour at all. Saponin also produces haemolysis with rise of viscosity (Table IV).

TABLE IV.

| Time of flow | |
|---|---|
| 64·4 secs. | before adding saponin, 0·020 g. |
| 90·0 „ | immediately after |
| 276·0 „ | five minutes later |

The rise is proportional to the amount of saponin added as is shown in Table V.

TABLE V.

| Time after adding saponin | Time of flow | |
|---|---|---|
| 0 | 33·5 secs. | 0·005 g. dry saponin added |
| 1½ mins. | 70·0 „ | |
| 4½ „ | 85·0 „ | |
| 20 „ | 83·0 „ | |
| 22 „ | | 0·005 g. dry saponin added |
| 35 „ | 115·0 „ | |

The viscosity is said by Burton-Opitz to be diminished by laking produced by freezing and thawing; but the condition which is most likely to be met with in the body is more akin to that of haemolysis by saponin.

## IV. MEASUREMENT OF THE CORPUSCULAR VOLUME.

The measurement of the volume of the corpuscles was carried out by centrifuging to a constant volume in a graduated tube, such as a haematocrit tube, with an ordinary electrical centrifuge working at 2500–3500 revolutions per minute. The graduated tube was closed by putting a piece of paper on the end and dipping into melted paraffin. A much firmer joint was thus made, than the leather pad and screw clip generally supplied. The time for complete sedimentation varies but is usually less than twenty-five minutes. The figures given in the following pages were obtained by centrifuging for fifty minutes. These measurements were necessarily made at room temperature, but by immersing the haematocrit tube in water at 40° it was found that the proportion between the volume of the corpuscles and of the plasma remained the same.

## V. THE CONTRIBUTION OF THE PLASMA TO THE VISCOSITY OF THE BLOOD.

The viscosity of the blood is directly proportional to that of the plasma, for the same volume of corpuscles, over a very wide range of plasma viscosity (Table VI and Fig. 2).

TABLE VI.

*Variation of the viscosity of suspensions of corpuscles with variation in the viscosity of an artificial plasma. Plasma:* 7 % *gum acacia in* 0·9 % *saline. Corpuscles: sheep, washed in saline.*

| Amount of suspension of corpuscles | 0·9 % saline | 7 % gum in saline | Viscosity | | $\frac{b}{p}$ |
|---|---|---|---|---|---|
| | | | plasma ($p$) | blood ($b$) | |
| 2 cc. | 1·5 cc. | 0·5 cc. | 1·40 | 3·22 | 2·30 |
| 2 „ | 1·0 „ | 1·0 „ | 1·77 | 4·06 | 2·29 |
| 2 „ | ·5 „ | 1·5 „ | 2·34 | 5·36 | 2·29 |
| 2 „ | 0 „ | 2·0 „ | 3·21 | 7·73 | 2·41 |

The table gives the result of increasing the viscosity of the plasma in a suspension of corpuscles in saline by the addition of gum acacia. Sheep's

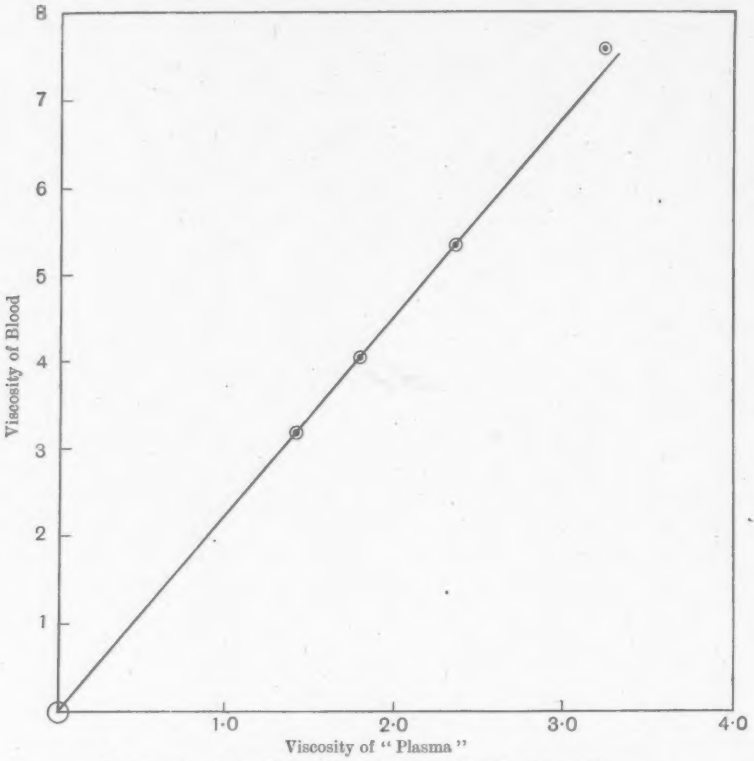


Fig. 2. Graph of relationship of viscosity of blood to that of plasma. The observed points are seen to lie along a straight line.

corpuscles were washed in 0·9 % saline and centrifuged until a fairly concentrated suspension was obtained. This was mixed with varying proportions of 0·9 % saline and 7 % gum acacia in 0·9 % saline as indicated in the table. The viscosity of the various mixtures was determined, as well as that of the "plasma" after the corpuscles had settled. The results are shown and the ratio between "plasma" viscosity and "blood" viscosity given. The constancy of the ratio is obvious while the values of the viscosity plotted against each other lie along a straight line intersecting the abscissa at 0. The value of the ratio $\frac{b}{p}$ (see Table VI) depends on the volume of the corpuscles present, the influence of which is next considered.

## VI. The Contribution of the Corpuscular Volume to the Viscosity.

The variation in the viscosity of the blood with the volume of the corpuscles present is shown in Table VII. Blood from various sources (indicated) was used, the viscosity of whole blood and plasma and the total volume of the corpuscles present were measured as before described.

Table VII.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|
| Source of corpuscles | Nature of "plasma" | Viscosity of "blood" comp. with water | Viscosity of "plasma" comp. with water | Viscosity of "blood" comp. with "plasma" | % corpuscular volume calculated from Formula 1 | % corpuscular volume found |
| Sheep | Saline | 30·50 | 1·00 | 30·50 | 91·0 | 90·5 |
| ,, | ,, | 15·05 | 1·00 | 15·05 | 78·9 | 79·1 |
| ,, | ,, | 11·13 | 1·00 | 11·13 | 74·5 | 73·5 |
| ,, | ,, | 7·10 | 1·00 | 7·10 | 63·5 | 62·5 |
| Dog | Plasma | (19·6)* | (3·00)* | 6·53 | 60·8 | 59·3 |
| ,, | ,, | 9·38 | 1·56 | 6·01 | 58·0 | 58·3 |
| ,, | ,, | 7·75 | 1·56 | 4·97 | 51·0 | 52·1 |
| Cat | ,, | 8·04 | 1·62 | 4·96 | 50·05 | 51·5 |
| Dog | ,, | 6·90 | 1·56 | 4·42 | 46·2 | 47·0 |
| ,, | ,, | 5·42 | 1·75 | 3·10 | 31·2 | 36·3 |
| Human | Plasma and citrate | 3·00 | 1·31 | 2·29 | 18·2 | 23·5 |
| ,, | ,, ,, | 2·30 | 1·31 | 1·76 | 7·9 | 11·75 |
| ,, | ,, ,, | 1·55 | 1·31 | 1·19 | 4·17 | 5·88 |

* Times of flow. Viscosimeter broken before calibration.

In column 7 of this table are given the haematocrit volumes and in column 6 a series of haematocrit volumes calculated from the formula

$$\frac{100}{P} = \left(\frac{n}{n-1}\right)^2 \quad \text{.....(1)}$$

where $P$ = volume of corpuscles as % of total volume of blood.

$n$ = the ratio of the viscosity of the whole blood to that of the plasma.

The agreement above a corpuscular volume of 45 % is within ± 1 % which is near the limit of error of the measurements. The curve *ACD* in Fig. 3 is the curve of the above equation and shows the agreement graphically.

The formula is one established mathematically by Hatschek [1912] for emulsions, when the volume of disperse phase is over 60 % and the rate of shear sufficient to convert the droplets of disperse phase into flat dodecahedra. The viscosity of the disperse phase does not appear in the formula, the only essential being that the disperse phase is easily deformable. This condition is fulfilled by the red corpuscles. The formula does not hold for emulsions below 60 % as at that point the droplets of disperse phase become spherical. In the case of blood corpuscles the discoid shape sufficiently approaches that of a dodecahedron for the formula to hold down to 45 %. Below this there

are other factors, including the rotational energy of the corpuscles, which become increasingly greater as the corpuscles become spaced out, and this, in its turn, causes the calculated and observed values to diverge more and more. The observed values below 40 % lie about a straight line $AB$ which is the graph of

$$n = 1 + \frac{KP}{100} \quad \text{.....(2)}$$

where $n$ and $P$ have the values previously assigned and $K$ is an arbitrary constant = approximately 6·3.

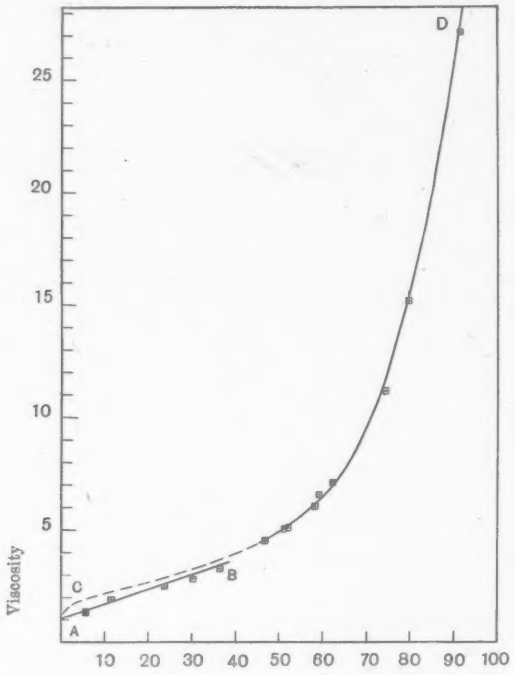


Fig. 3. Relation of viscosity to corpuscular volume.
⊡ observed points.
$ACD$ = graph of formula 1.
$AB$ = graph of formula 2.

This formula is similar to that deduced mathematically for suspensions of spheres by Hatschek [1912]

$$n = 1 + \frac{4{\cdot}5P}{100} \quad \text{.....(3)}$$

but differs in the constant $K$ which, as Hatschek pointed out, depends on the shape of the suspended body. 6·3 was selected as most nearly representing the observed figures. The calculation of the constant for a body of the shape of the corpuscle presents considerable difficulty.

## VII. Discussion.

1. The relationships established above will perhaps be made clear by the following. In narrow passages the blood corpuscles when crowded together move edge foremost. Their distribution next to the wall of the capillary can be represented by the diagram in Fig. 4. The layer of corpuscles next the wall ($A$) is practically stationary. That next, at $B$, is travelling slowly, that at $C$ a little faster and so on. The internal friction is produced between the layers of plasma and the surface of the corpuscles: the total kinetic energy of the corpuscles is the same at the beginning and the end of the capillary tube so that the energy degraded into heat in the tube is due entirely to the internal friction of the plasma moving relatively to the corpuscles. This being the case, the conditions can be made clearer by treating the corpuscles as fixed and the plasma as moving along the chinks between them with varying velocities, slowest near the wall and greatest in the centre of the tube. The rate of flow of fluids along such narrow passages was shown by Poiseuille to be inversely proportional to the viscosity. Hence the influence of the plasma on the viscosity of the blood is explained.

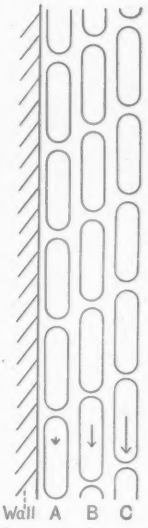


Fig. 4. Diagram of the flow of corpuscles along the wall of the capillary viscosimeter. The arrows represent by their direction and length the direction and velocity of each row of corpuscles.

He also showed that the rate of flow of fluids along narrow capillary passages is proportional to the fourth power of the radius of the capillary. This explains why the increase of the volume of the corpuscles present, which narrows the passages along which the blood flows, produces such a markedly increasing influence on the viscosity of the blood.

2. These experiments suggest a few conjectures as to the shape-consistency of the red corpuscles. Any evidence available goes to show that other living cells are more or less fluid and tend therefore to assume a spherical shape when freed from constraining influences, or unless they are

definitely amoeboid; *e.g.* in the numerous cultural experiments with living tissue cells [N. C. Lake, 1916] the growing cells which are found in such cultures are nearly always spherical whatever their origin, unless they become adherent to the lymph or other solid culture medium used. But the red corpuscles, although still undergoing metabolic changes (consumption of $O_2$, glucose [see MacLean 1916] *et alia*) are flat discs and therefore rigid, if elastic. Even the nucleated corpuscles of birds etc. are flattened. Now consider for a moment what would happen if the red corpuscle were a sphere of liquid protoplasm such as the white corpuscle. If the total volume of corpuscles present were less than 60 % the spheres would be completely separated by plasma, but at 60 % the corpuscular volume would be so great as to cause the spheres to come in contact. Up to that point the viscosity would increase almost in a straight line relationship with the increase in volume, but above it two things may happen depending on the velocity of flow. If the velocity of flow is insufficient to deform the spheres into flat discs the corpuscles would "jam" and cease to flow through the capillaries or only flow exceedingly slowly. Plasma would still flow through the interspaces. Such a condition as this however is much more rarely set up with disc-shaped corpuscles.

3. The white corpuscles have been included in the total volume of corpuscles measured in the above experiments. The rate of shear in the capillary viscosimeters used is probably sufficient to produce in them the requisite change of shape, but in any case their relative volume in most cases is so small as not to affect the results appreciably. In conditions such as myelocytic leukaemia, however, the relative volume of the white corpuscles is so large that considerable modifications of the conditions of capillary flow must arise, and it was hoped, but in vain, that this question of the influence of white cells on the viscosity might have been investigated before publishing these figures. It will at once occur to the reader that the amoeboid properties of the white cell would prevent the block in the capillaries becoming absolute, and that therefore the circulation of dead and of living leucocytes would produce different results.

4. These experiments suggest one or two observations as to the microscopic vascular changes associated with inflammation and infarction. The first stage in the vascular change accompanying inflammation is a dilatation of the capillaries accompanied by an increased rate of flow: this is succeeded by an increased permeability of the capillary wall leading to an increased outpouring of lymph: the rate of flow of the blood then gradually slows down

and finally almost ceases. It is suggested that this stage of stasis in inflammatory conditions is due not to any special change in the wall but to the very great and increasing rise in the viscosity of the blood consequent on the loss of plasma. This condition of stasis would be commoner than it is were the corpuscles not flattened discs.

SUMMARY.

1. A form of capillary viscosimeter for blood is described in which stirring during the observations is possible.

2. Errors and precautions during the measurement are discussed.

3. The viscosity of the blood is shown to be a constant multiple of that of the plasma for the same number of corpuscles.

4. Mathematical relationships are shown to hold between the number of corpuscles and the viscosity.

5. Some of the bearings of the results are discussed.

The work was carried out during the tenure of a grant from the Medical Research Committee to whom my thanks are due.

I have to thank Professor Bainbridge for much kind encouragement and criticism during the progress of the work.

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# V. ON β-CHOLESTANOL, SOME OF ITS DERIVATIVES AND OXIDATION PRODUCTS.

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Gardner and Godden [1913] showed that coprostanone on oxidation by chromic acid yielded a small quantity of an acid, melting at 247°, identical with that obtained as a bye-product in the preparation of coprostanone from coprosterol. On oxidation by ammonium persulphate the coprostanone yielded as main products two isomeric substances of the formula $C_{27}H_{46}O_2$, melting respectively at 183–184° and 157–158°. These appeared to be lactones of a hydroxyacid or hydroxyacids of the formula $C_{27}H_{48}O_3$. It seemed possible that light might be thrown on the relationship of coprosterol to the various isomeric artificial bihydrocholesterols—ψ-coprosterol, β-cholestanol, etc.—by a study of their oxidation by means of ammonium persulphate. We therefore commenced our experiments with β-cholestanol but the work has been much delayed by the absence of one of us on more urgent duties.

In the meantime Windaus and his co-workers have very materially increased our knowledge of this group of alcohols. Uibrig and Windaus [1913, 1914, 1915] working on the four alcohols, (1) β-cholestanol, (2) ε-cholestanol, (3) coprosterol, and (4) ψ-coprosterol, showed that β-cholestanol and ε-cholestanol can be changed into one another by boiling in amyl alcohol solution with sodium amylate, and differ from one another only in the stereo-position of the hydroxyl group with regard to an asymmetric carbon atom, as in the case, for instance, of borneol and iso-borneol. A similar relationship obtains between coprosterol and ψ-coprosterol, as had been previously pointed out by Dorée and Gardner [1908]. β-Cholestanol and coprosterol, they maintain, differ from one another in the stereo-position of a hydrogen atom with regard to another asymmetric carbon atom, but they had not been able to change the one into the other. The relationship of ε-cholestanol to ψ-coprosterol is similar.

In a later paper Windaus [1916] gave an account of the reduction of

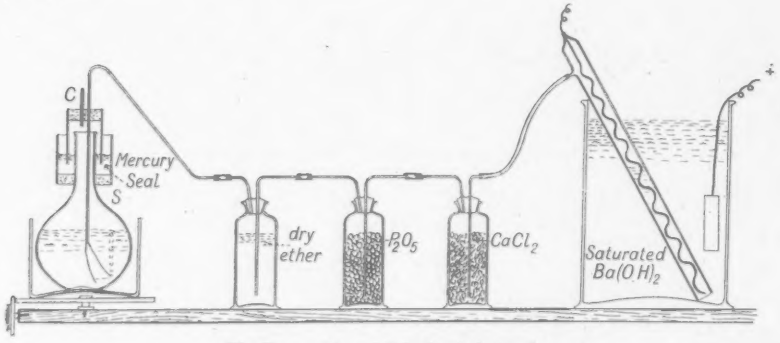
cholesterol by means of hydrogen at 200° in the presence of reduced nickel and described a new alcohol, γ-cholestanol, M.P. 146° and $[\alpha]_D^{18} + 29{\cdot}9°$. This he found to be really a conglomerate of β-cholestanol, ψ-coprosterol and ε-cholestanol in the sense of the partial racemate compounded of β-cholestanol and ψ-coprosterol. They crystallise together like a single compound and the product, γ-cholestanol, has a similar melting point and similar specific rotary power to β-cholestanol, the only difference being that γ-cholestanol crystallises from dilute alcohol in the anhydrous condition, whereas β-cholestanol crystallises with one molecule of water. The separation of the constituents of γ-cholestanol was effected by taking advantage of the fact that, whereas β-cholestanol and coprosterol are precipitable by digitonin, the isomers ψ-coprosterol and ε-cholestanol do not form insoluble compounds with this substance. The β-cholestanol was removed by means of digitonin and the isomerides partially separated by crystallisation from methyl alcohol. ε-Cholestanol separates first and the part which remains in the crude ψ-coprosterol is re-arranged into β-cholestanol by boiling the mixture with sodium and xylene. This β-cholestanol was again precipitated by digitonin and the pure ψ-coprosterol obtained. Finally, Windaus found that the ψ-coprosterol could be partly transformed into the isomeric coprosterol by heating with alcoholic sodium ethoxide at 180°. The coprosterol was separated from the mixture by precipitating with digitonin and recovered from the digitonide in the usual manner by boiling with xylene. From the provisional formulae proposed by Windaus we should expect the oxidation products of β-cholestanol and β-cholestanone to be very similar, if not identical, with those obtained by Godden and Gardner from coprosterol and coprostanone. The dicarboxylic acid, melting at 247°, obtained from coprosterol by Godden and Gardner and by J. Adamla [1911] from γ-cholestanol, we did not find among the oxidation products of either β-cholestanol or β-cholestanone.

The β-cholestanone was less readily attacked by ammonium persulphate than coprostanone and the products obtained, though apparently lactones of the formula $C_{27}H_{46}O_2$, were different from those prepared from coprostanone and were more easily hydrolysed. They were very difficult to separate and the behaviour of the lower melting isomers suggested either that different isomers possessed the property of crystallising together like single substances, as Windaus has shown for different bihydrocholesterols, or that different isomers are readily re-arranged one into the other. None of the substances obtained was, however, identical with any of those got from coprosterol.

## Experimental.

*Preparation of β-cholestanol.* The reduction of cholesterol to β-cholestanol was first carried out by Willstätter and Meyer [1908] by the use of hydrogen and platinum black in ethereal suspension. The exact technique of this method has been somewhat modified by us and with such good results that it may therefore be described. The dry pure ether used was prepared from the commercial product by treatment in the cold with about 10 % of its weight of a finely powdered mixture of dry potassium hydroxide and potassium permanganate in the proportion of about four parts of potash to one of permanganate. The mixture was occasionally shaken for a period of 24–28 hours and after settling the ether was decanted and distilled over phosphorus pentoxide.

The hydrogen, which it is essential should be pure and dry, was prepared electrolytically, the electrolyte employed being a saturated solution of barium hydroxide. The cathode was a silver coil and the anode a platinum coil. The general arrangement of the voltameter is shown in the accompanying diagram.




The Preparation of Dihydrocholesterol.

The hydrogen passed through a calcium chloride tower, through phosphorus pentoxide and then through dry ether. The latter served the purpose of arresting any traces of phosphorus pentoxide and of making up for loss of ether from the cholesterol solution by its own evaporation. The ethereal solution of cholesterol, which should be not more than three-fourths saturated, contained about 8 grams of platinum black to 400 cc. of the solution. The platinum black was prepared according to the method of Loew [1890].

It was found very necessary to keep the solution of cholesterol continually and vigorously shaken, in order that the heavy platinum black should be in

a continual state of suspension. We believe this suspension can be more completely established by the method shown in the figure. The flask containing the solution is kept in rapid rotation; the stirrer is fixed, but is composed of a bent, wide-mouthed tube, so arranged as to stir up the rotating ether. Such a movement can be readily obtained by mounting a flask in a glass dish by means of four corks and mounting the dish on a horizontal wheel rapidly rotated by means of a pulley coupled with a motor. Such a device was found completely to reduce 50 grams of cholesterol in 24 hours. The evaporation of the ether is avoided largely by the introduction of the ether wash bottle, reducing the outlet of the flask by the use of a mercury valve *S* and allowing the gas to escape by a capillary tube *C*. The completion of reduction is best ascertained by an estimation of the specific rotary power which is the reverse of that of cholesterol.

The rotation was found to be $[\alpha]_D^{18} + 28{\cdot}8°$, a value agreeing with that given by Willstätter and Meyer.

A number of esters were prepared at the beginning of our work with a view of comparing their properties with those of other isomers; we give here the properties of the chloroacetate, benzoate, and stearate which as far as we are aware do not appear to have been fully described.

*β-Cholestanol chloroacetate.* Two grams of β-cholestanol were dissolved in 20 cc. chloroform which had been previously freed from alcohol by washing, and then dried, and distilled. One gram of chloroacetyl chloride was added and the mixture gently boiled under a reflux for a few minutes. Another gram of the reagent was then added and the boiling continued for 30 minutes. The solution was then evaporated and the residue taken up in ethyl acetate, from which, on cooling, crystals were readily obtained. They were purified by recrystallisation from a mixture of chloroform and ethyl acetate. The yield was approximately theoretical. β-Cholestanol chloroacetate crystallises in glistening plates not unlike cholesterol in appearance and melts at 178–179°. It is readily soluble in ether and in chloroform but only slightly so in cold ethyl acetate.

Analysis: 0·2321 g.; 0·2210 $H_2O$; 0·6361 $CO_2$.

Found C = 74·15, H = 10·58.

Calculated for $C_{29}H_{49}ClO_2$ C = 74·92, H = 10·54.

*β-Cholestanol benzoate.* 1·5 grams of β-cholestanol were dissolved in 15 cc. of pure dry pyridine and 2·5 cc. of benzoyl chloride added. The mixture was allowed to stand overnight, poured into about 50 cc. of water and the precipitated benzoate filtered off and washed. It was crystallised from

a mixture of benzene and acetone and finally from ethyl acetate and was thus obtained in the form of small plates. The yield was satisfactory. It is only slightly soluble in cold alcohol and not very soluble in hot. It is fairly soluble in ether, readily in benzene, and slightly so in acetone. It melts at 155° and during the melting process displays an extraordinarily brilliant fluorescence. On gently heating in a melting point tube it assumes at about 138–139° a reddish tinge, and at 140° begins to soften and run together to an opaque opalescent mass of a red tinge with flashes of green. At 145° it is still opaque and the play of colours becomes more intense, red and emerald green predominating according to the point of view. The fluorescence becomes brighter as the temperature rises, until at 155° the colours suddenly vanish and the liquid becomes clear. On cooling, the liquid becomes opaque again at 155° and the colour display appears again in the reverse order. The colours are not shown in transmitted light. The colour display may be very strikingly demonstrated by heating between two microscope slides. Owing to this colour display the exact melting point could not be determined.

In chloroform solution the rotation was found to be $[\alpha]_D^{23} + 23{\cdot}3°$.

Analysis: 0·2222 g.; 0·21 $H_2O$; 0·6725 $CO_2$.

Found C = 82·54, H = 10·50.

Calculated for $C_{34}H_{52}O_2$, C = 82·92, H = 10·57.

*β-Cholestanol stearate.* An attempt to obtain this substance by a process similar to that employed for the preparation of the benzoate was not successful, only a very small percentage yield being obtained. A good yield was obtained by adding to 1 gram of the β-cholestanol about twice its weight of stearyl chloride, the whole being in chloroform solution. The solution was left to stand in a closed flask for a day, then boiled gently for half-an-hour and finally evaporated to dryness. This stearate was almost insoluble in alcohol, but soluble in a mixture of alcohol and chloroform, from which it readily crystallised in soft flaky crystals. It melts at 100° and is readily soluble in ether. The rotation in ether solution was $[\alpha]_D^{20} + 18{\cdot}4°$.

Analysis: 0·1599 g.; 0·1869 $H_2O$; 0·4856 $CO_2$.

Found C = 82·82, H = 12·99.

Calculated for $C_{45}H_{82}O_2$, C = 82·57, H = 12·54.

*Oxidation of β-cholestanol.* Diels and Abderhalden [1906] first showed that β-cholestanol is readily oxidised to a ketone, β-cholestanone, by the action of chromic acid in acetic solution. This ketone melts at 128–129°, whereas coprostanone melts at 62–63°. In the preparation of coprostanone from coprosterol Godden and Gardner found that the yield never exceeded

70 %, and the ketone was accompanied by considerable quantities of a dibasic acid, $C_{27}H_{46}O_4$ melting at 247°, and smaller amounts of a non-crystalline acid.

In our experiments on the preparation of β-cholestanone the yield was from 80–90 %. The glacial acetic solution of the oxidation product was diluted with water and extracted with ether. On shaking the washed ethereal solution of the ketone with dilute aqueous potash a trace of a crystalline acid was obtained on acidifying the alkaline extract. The amount of this acid appeared to be slightly increased by conducting the oxidation in more gentle fashion. We did not obtain it in sufficient quantity for further investigation, but it was quite different from the above mentioned dibasic acid, M.P. 247°, obtained from coprosterol.

*Oxidation of β-cholestanone by ammonium persulphate.* This oxidation was carried out in a manner somewhat similar to that described by Godden and Gardner in the case of coprostanone, but took place less readily. Four grams of the ketone were dissolved in 150 cc. of glacial acetic acid and 4 grams of ammonium persulphate in 8 cc. of water were added. The mixture was placed on a water bath for an hour and left over-night at a temperature of about 80°. The next day another 4 grams of the persulphate were added in small amounts and the mixture allowed to stand for 4 days at 80°. On cooling crystals separated and were filtered off. The filtrate was then diluted and extracted with ether. This extract was added to an ethereal solution of the crystals already separated and the whole extracted by shaking with a solution of sodium carbonate. This extract on acidification yielded a trace only of an oil. The ethereal solution was then further extracted with 10 cc. potash. The ether solution thus extracted was found to contain a small amount of unchanged ketone. Several oxidations were carried out in this way. The potash extract on acidification with hydrochloric acid yielded a flocculent precipitate, soluble in alcohol, from which it crystallised in clots of fine needles. These were recrystallised from ethyl acetate and melted at about 180°. On dissolving the crystals in ether and extracting with potash it was found that now very little of the substance was removed. On evaporating the ether and recrystallising the residue from alcohol crystals melting round 180° were again obtained. This behaviour recalls the properties of the lactones from coprostanone described by Godden and Gardner. The crystals were readily soluble in ether, alcohol, or ethyl acetate, and when allowed to cool after melting in a capillary tube showed an opalescent appearance and after 30 seconds the contents of the tube suddenly gave a slightly

audible crackle. This phenomenon, by remelting, could be repeated indefinitely.

This crystalline matter was fractionally crystallised from various solvents —alcohol, acetone, ether and methyl alcohol. A fraction separated from alcohol in clots of microscopic needles. This, after repeated recrystallisation from alcohol and from acetone melted sharply at 201–202°.

Analysis: 0·1034 g.; 0·10655 $H_2O$; 0·30585 $CO_2$.

Found C = 80·67, H = 11·45.

Calculated for $C_{27}H_{46}O_2$, C = 80·52, H = 11·50.

It was not attacked by acetic anhydride and had no ketonic properties. It was not hydrolysed by shaking the ethereal solution with potash, strong or dilute, but on boiling with alcoholic soda it yielded a salt soluble in water. On acidification the substance was re-formed and after recrystallisation from alcohol melted at 201–202°. It was presumably therefore a lactone of an acid $C_{27}H_{48}O_3$.

In addition to this lactone a small quantity of a body melting at 184–186° was obtained, the solubility of which was of the same order. This crystallised from alcohol in thin plates or needles very similar in appearance to the substance of the same melting point obtained by Godden and Gardner from coprostanone. It was not, however, identical, as on mixing the two substances the product melted between 160° and 170°. The amount of the pure substance was too small for combustion or further examination.

The crystalline matter left after the separation of these two bodies consisted of small needles and had the appearance of a single substance. Its behaviour on melting however showed that this was not the case, as it gradually softened through a considerable range of temperature. After repeated fractionation the following fractions were obtained:

A. A very small fraction melting at 98–100°. On combustion this was found to contain 80·5 % of carbon and 12·2 % of hydrogen. It may possibly be a low-melting lactone $C_{27}H_{46}O_2$, but the quantity was too small for further investigation.

B. Fine matted needles, which on heating began to shrink together at 144°, softened and melted at 152–153° and ran to a clear liquid at 157–158°.

C. Indeterminate crystalline matter. On heating it began to shrink together at 120° and melted at 130° to a turbid liquid, which became clear at 138–139°. This behaviour was shown again on cooling and re-heating.

D. Small needle-like crystals. It began to soften at 143°, softened at 150° and ran between 159° and 161° to a clear liquid. On cooling long needles

began to form in the clear liquid at 158–159°, but the whole mass did not become opaque until a much lower temperature. On re-heating it became clear again at 150°, but the needles did not vanish until 159–161°. Further recrystallisation did not alter the melting point materially.

Analysis:

(1) 0·1005 g.; 0·1013 $H_2O$; 0·2946 $CO_2$.

(2) 0·0894 g.; 0·0915 $H_2O$; 0·2633 $CO_2$.

| | | C | H |
|---|---|---|---|
| Found | (1) | 79·94 | 11·20 |
| | (2) | 80·32 | 11·50 |
| Calculated for $C_{27}H_{46}O_2$, | | 80·52 | 11·50. |

This was the main fraction. It was different in properties from the substance of similar melting point obtained from coprostanone. On boiling with weak alcoholic soda it yielded a sodium salt which dissolved in water to a soapy solution. On acidification the substance was not re-formed again, but a pasty precipitate was obtained. We had not sufficient for further examination of this acid. The substance was evidently not pure, but consisted mainly of a lactone or mixture of lactones, of the above formula.

*Fraction C* was analysed.

0·1119 g.; 0·1134 $H_2O$; 0·3366 $CO_2$.

Found C = 82·04, H = 11·26.

It was thought that perhaps this might contain some unchanged ketone, or even unchanged β-cholestanol, but mixed meltings did not seem to bear this out.

*Fraction B* was also a mixture and on combustion was found to contain 81·7 % of carbon and 11·44 % of hydrogen.

It was thought that if these intermediate fractions consisted of mixtures of high and low melting lactones it might be possible to destroy the lower melting ones by further oxidation with chromic acid, as Godden and Gardner had found that this was the case with similar mixtures from coprostanone. The results were negative and crop C gave a product of more or less unchanged melting point.

It would appear from these results that β-cholestanone on oxidation with persulphate yields a mixture of lactones of the formula $C_{27}H_{46}O_2$, probably two of high melting point and one or more of lower melting point, but as far as we were able to ascertain they were different from those obtained from coprostanone.

We hope later on to be able to give a more complete account of these various substances.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for assistance in carrying out this work.

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# VI. SUR LA FERMENTATION DE L'ACIDE GLYOXYLIQUE.

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(*Received January 23rd, 1918.*)

EN étudiant l'action de la levure, préparée d'après ma méthode, sur quelques acides organiques, j'ai fait aussi des expériences sur l'acide glyoxylique. Celui-ci, ayant une fonction d'aldéhyde, présente sûrement un intérêt particulier. L'action de la levure vivante sur l'acide glyoxylique a été examiné par Neuberg et Tir [1911], mais le résultat a été négatif: de toutes les races employées par ces auteurs une seulement (la race A) a donné un peu plus de $CO_2$ que le témoin (7 cc. de $CO_2$ contre 1·5 cc.) dans 2 tubes de Schrotter.

D'après mes expériences il faut admettre que la levure de Lebedev peut décomposer l'acide glyoxylique avec dégagement de $CO_2$. Il n'est pas facile pour le moment d'expliquer de quelle manière agit cette réaction curieuse. A peine pourrait-on supposer que le dégagement de $CO_2$ soit dû tout simplement à l'excitation de l'autofermentation, parce que le même phénomène a lieu lorsqu'on emploie du suc de macération. Parallèlement au dégagement de $CO_2$ il se forme une quantité notable de $CH_3COH$. Si l'on se souvient de ce qu'on peut obtenir l'acide glyoxylique par l'oxydation de $C_2H_6O$, ce ne serait peut-être pas trop osé d'admettre que la levure puisse réduire celui-là en le transformant en $C_2H_6O$, étant donné que l'aldéhyde acétique lui-même se transforme en partie sous l'action de la levure en alcool éthylique.

*Exp.* 1. On a pris 6 fioles d'Erlenmeyer avec une soupape de fermentation de Meissl (Nos. 1–6). Elles ont été chargées de 1 gr. de levure + 50 cc. d'eau + 0·2 cc. de toluène. Aux Nos. 1–4 on a ajouté 100 mg. d'acide glyoxylique à chacune.

Température 25°.

| t. | No. 1 | No. 2 | No. 3 | No. 4 | Moyen | Moyen des Nos. 5 et 6 |
|---|---|---|---|---|---|---|
| Après 17 h. (mg. de $CO_2$) | 112 | 114·5 | 104·5 | 115 | 111·5 | 85·5 |
| „ 45 „ „ | 145 | 150·5 | 134·5 | 159 | 147·0 | 114·5 |
| „ 65 „ „ | 154 | 161·0 | 142·0 | 178 | 159·0 | 123 |

Alors on obtient par soustraction: 111·5 – 85·5 = 26 mg.; 147 – 114·5 = 32·5 mg.; 159 – 123 = 36 mg. de $CO_2$ pour chaque fiole.

*Exp.* 2 est analogue à la précédente.

| t. | No. 1 | No. 2 | No. 3 | No. 4 | Moyen | No. 5 | No. 6 | Moyen |
|---|---|---|---|---|---|---|---|---|
| 4 h. | 20·5 | 18·5 | 27 | 19·0 | 21·0 | 24 | 16 | 20·0 |
| 8 h. | 66·5 | 72·5 | 72 | 71·5 | 70·5 | 50 | 39 | 44·5 |
| 24 h. | 101·0 | 104·5 | 100 | 101·5 | 102·0 | 84 | 63 | 73·5 |

21 – 20 = 1 mg.; 70·5 – 44·5 = 26 mg.; 102 – 73·5 = 28·5 mg. de $CO_2$ pour chaque fiole.

Dans les Nos. 3 et 4 et dans les témoins (Nos. 5 et 6) on a dosé $CH_3COH$ en forme de sa *p*-nitrophénylhydrazone sans la recristalliser (F. 127°). On a mélangé le contenu des Nos. 3 et 4 et des Nos. 5 et 6; de cette manière on a obtenu 2 portions de liquide; on les a neutralisées soigneusement et on en a fait distiller $\frac{1}{3}$ de volume, en refroidissant le récipient avec de la glace fondante. Dans ces conditions il ne passe que des traces d'acide glyoxylique comme cela a été prouvé par une expérience préliminaire. On a obtenu 35 mg. de hydrazone (F. 127°); le témoin au contraire n'en a rien donné.

*Exp.* 3. On a fait fermenter l'acide glyoxylique avec du sucre (pour 100 mg. d'acide 200 mg. de sucre) et on a dosé $CH_3COH$. T = 25°.

| t. | ac. glyox. + sucre | | ac. glyoxylique | | sucre | | témoins | |
|---|---|---|---|---|---|---|---|---|
| 12 h. | 147 | 156 | 87 | — | 136 | 134 | 78·5 | 69·5 |
| 33 h. | 174 | 178 | 108 | — | 166 | 178 | 97·5 | 88·0 |
| 47 h. | 209 | 208 | 130·5 | 134 | 190 | 218 | 118·5 | 107·0 |
| | 32 mg. d'hydrazone | | 30 mg. d'hydrazone | | 8 mg. d'hydrazone | | traces | |

*Exp.* 4. On a pris 4 fioles et on les a chargées chacune de 40 cc. de suc de macération; 2 fioles ont été additionnées chacune de 62·5 mg. d'acide. On a ajouté, comme toujours, à chaque fiole du toluène. T = 25°.

| t. | ac. glyoxylique | | témoins | |
|---|---|---|---|---|
| 21 h. | 9 | 12·5 | 4 | 4·6 |
| 45 h. | 19 | 22·5 | 7 | 6·5 |
| 69 h. | 27 | 27·5 | 8 | 8·0 |

Le suc qui a été chauffé pendant 10 min. à 70° n'agissait pas sur l'acide glyoxylique.

*Exp.* 5. On a pris 4 fioles avec des soupapes de fermentation; à chacune d'elles on a ajouté 22·5 cc. de suc. Aux 2 fioles on a ajouté en outre 2·5 cc. de solution à 3·54 % d'acide glyoxylique à chacune (88·5 mg.).

| t. | $CO_2$ (ac. glyoxylique) | | $CO_2$ (témoins) | |
|---|---|---|---|---|
| 48 | 13·5 | 16 | 6 | 6 |
| 72 | 20·5 | 25 | 11 | 9 |

29·5 – 12 = 17·5 mg.; 45·5 – 20 = 25·5 mg. Pour une fiole: 9 et 13 mg. de $CO_2$.

*Exp.* 6. 4 fioles ont été chargées de la levure qu'on a conservée au laboratoire au moins pendant une année. Pour 10 gr. de levure on a pris 50 cc. d'eau. Aux 2 fioles on a ajouté en outre 100 mg. d'acide glyoxylique à chacune. T = 25°.

| t. | $CO_2$ (ac. glyoxylique) | | $CO_2$ (témoins) | |
|---|---|---|---|---|
| 11 h. | 48·5 | 50·5 | 50·0 | — |
| 24 h. | 100·5 | 105·5 | 98·5 | 105 |
| 29 h. | 113·5 | 118·0 | 114·5 | 119 |

Si l'on considère la quantité de $CO_2$ dégagé, on ne s'aperçoit pas d'une différence quelconque entre les fioles chargées de $CHO . CO_2H$ et les témoins, néanmoins on a obtenu 28 mg. d'hydrazone qui, recristallisée une fois, avait le point de fusion 128°. Le témoin n'a donné après la distillation aucune hydrazone. Ce fait curieux dit en faveur de la supposition que l'aldéhyde acétique se forme à cause de la réduction de l'acide glyoxylique. Pour faire ressortir plus clairement l'influence de la concentration, j'ai fait l'expérience suivante.

*Exp.* 7. On a pris 5 fioles (Nos. 1–5) avec des soupapes de fermentation; on a ajouté 10 gr. de levure, 50 cc. d'eau et 0·2 cc. de toluène à chacune. Aux fioles Nos. 1, 2 et 3 on a ajouté en outre 300, 200 et 100 mg. d'acide glyoxylique. T = 25°.

| t. | 300 mg. d'ac. glyox. | 200 mg. d'ac. glyox. | 100 mg. d'ac. glyox. | témoins | |
|---|---|---|---|---|---|
| 21 h. | 19 | 40 | 73 | 65 | 33·5 |
| 27 h. | 30 | 70 | 109 | 94 | 69·5 |
| 39 h. | 40 | 84 | 137 | 105 | 99·5 |
| 64 h. | 40 | 84 | 144 | 120 | 121·0 |

Le mécanisme de la décomposition n'étant pas bien clair, je me suis posé la question si l'acidité du milieu influencerait l'autofermentation d'une manière sensible.

*Exp.* 8. 2 fioles ont été chargées de 10 gr. de levure, 50 cc. d'eau et 0·2 cc. de toluène. A une d'elles on a ajouté 0·1 cc. de HCl.

| t. | HCl | témoin | différence |
|---|---|---|---|
| 3 h. | 50 mg. de $CO_2$ | 21 mg. de $CO_2$ | 29 mg. de $CO_2$ |
| 20 h. | 112 „ „ | 63 „ „ | 49 „ „ |
| 28 h. | 126 „ „ | 72 „ „ | 54 „ „ |
| 55 h. | 156 „ „ | 113 „ „ | 43 „ „ |
| 70 h. | 191 „ „ | 166 „ „ | 25 „ „ |

Ainsi devient-il plausible que l'acide minéral puisse favoriser l'autofermentation de la levure, il faut donc s'attendre à ce que les acides organiques puissent produire, suivant le degré de leur dissociation électrolytique, le même effet.

Les expériences avec les acides glycolique, acétique, lactique, etc. m'ont donné un résultat nettement positif. J'en ferai connaître quelques-unes faites à ce sujet.

*Exp.* 9. 4 fioles ont été chargées chacune de 10 gr. de levure et 50 cc. d'eau. Aux 2 fioles on a ajouté 200 et 100 mg. d'acide glycolique.

| t. | 200 mg. | 100 mg. | témoins | |
|---|---|---|---|---|
| 17 h. | 70 | 76 | 66·5 | 59·0 |
| 24 h. | 94 | 95 | 80·5 | 77·5 |
| 38 h. | 130 | 139 | 125·5 | 124·5 |
| 43 h. | 146 | 168 | 154·0 | 153·0 |

*Exp.* 10. Au lieu de 10 gr. de levure on a pris 25 cc. de suc de macération. Aux 2 fioles on a ajouté 62 mg. d'acide glycolique à chacune.

| t. | ac. glycol. | | témoins | | différence pour 1 fiole |
|---|---|---|---|---|---|
| 14 h. | 9 | 12 | 4 | 4·0 | 6 mg. de $CO_2$ |
| 45 h. | 19 | 22 | 7 | 6·5 | 14 „ „ |
| 69 h. | 27 | 28 | 8 | 8·0 | 20 „ „ |

Cette expérience dit plutôt en faveur de la supposition que l'acide glycolique soit décomposé sous l'influence de la levure. Je n'ai pas réussi à préciser définitivement quelle était l'action de HCl sur le suc de levure, parce qu'une petite quantité d'acide minéral suffisait déjà pour en faire une coagulation partielle.

*Exp.* 11. On a pris 6 fioles chargées chacune de 10 gr. de levure + 50 cc. d'eau + 0·2 cc. de toluène (Nos. 1–6). Aux fioles 1 et 2 on a ajouté 378 mg. d'acide glycérique, aux fioles 3 et 4 209 mg. d'acide acétique à chacune.

| t. | ac. glycér. | | ac. acét. | | témoins | | différence pour 1 fiole | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | ac. glycér. | ac. acét. |
| 28 h. | 166 | 183 | 117 | 116 | 66 | 67 | 108 mg. de $CO_2$ | 50 mg. de $CO_2$ |
| 55 h. | 188 | 166 | 166 | 158 | 102 | 112 | 70 „ „ | 55 „ „ |

*Exp.* 12. Cette expérience est analogue aux précedentes; aux 2 fioles on a ajouté 200 mg. d'acide lactique à chacune.

| t. | ac. lactique | | témoins | | différence pour 1 fiole |
|---|---|---|---|---|---|
| 28 h. | 109 | 121 | 66 | 61 | 51·5 mg. de $CO_2$ |
| 49 h. | 132 | 150 | 104 | 91 | 43·5 „ „ |
| 72 h. | 139 | 160 | 138 | 118 | 21·5 „ „ |

Elle montre que la différence diminue avec le temps, ce qui donne tout lieu à croire que le dégagement de $CO_2$ en présence de l'acide lactique était dû plutôt à l'action stimulante exercée par lui qu'à la décomposition de l'acide lactique, d'autre part les expériences que j'ai publiées antérieurement ont démontré [Lebedev, 1915, 1916] que la présence de l'acide lactique se manifestait seulement par l'augmentation de la quantité de $CO_2$ dégagé, mais pas

d'alcool formé; le rapport $\frac{CO_2}{C_2H_6O}$ pendant l'autofermentation reste au contraire presque égal à l'unité. On voit de tout ce que je viens de dire qu'il n'est pas facile de se faire une idée précise de ce qui se passe en vérité et il faudra encore beaucoup d'expériences pour y mettre une pleine clarté. La fermentation de l'acide glycérique a été établie par moi [Lebedev, 1914] fermement, parce que le dégagement de $CO_2$ était accompagné de la formation de $CH_3COH$. Dans le travail fait par moi[1] en collaboration avec M. Polonsky nous avons dosé tous les produits qui se formaient pendant la décomposition de l'acide glycérique, à savoir $CO_2$, $CH_3.COH$, $CH_3.CH_2OH$ et $CH_3.CO_2H$, et nous avons constaté que la somme $(CH_3.COH + CH_3.CO_2H + CH_3.CH_2OH)$ était presque égale à la quantité de $CO_2$. En ce qui concerne les acides glyoxylique, acétique, lactique et glycolique la question reste ouverte, parce que, excepté $CO_2$ et $CH_3.COH$, on n'a pas découvert, tout au moins momentanément, d'autres produits de leur décomposition.

Il était intéressant de voir quelle action exerçait un milieu neutre sur le travail de la levure de Lebedev pendant la fermentation du sucre, c'est pourquoi j'ai fait quelques expériences en présence de $CaCO_3$; je vais reproduire ici une de celles-ci.

*Exp.* 13. 4 fioles chargées chacune de 10 gr. de levure + 50 cc. d'eau + 0·2 cc. de toluène. On a ajouté au No. 1—5 gr. de sucre, au No. 2—5 gr. de sucre + 1 gr. de $CaCO_3$, au No. 3—1 gr. de $CaCO_3$.

| | 1 | 2 | 4 | 3 | | | |
|---|---|---|---|---|---|---|---|
| t. | sucre | sucre + $CaCO_3$ | témoin | tém. + $CaCO_3$ | No. 1 – No. 4 | No. 2 – No. 3 | (1 – 4) – (2 – 3) |
| 24 | 1173 | 879 | 82 | 69 | 1091 | 810 | 281 |
| 27 | 1379 | 1048 | 156 | 119 | 1223 | 929 | 294 |
| 48 | 1447 | 1058 | 229 | 162 | 1218 | 896 | 322 |

On a dosé $CH_3.COH$ et on a obtenu: No. 1—5·5 mg., No. 2—10·5 mg. de *p*-nitrophénylhydrazone, Nos. 3 et 4—des traces de celle-ci.

On peut expliquer l'action de $CaCO_3$ de trois manières. 1. Le milieu neutre est nuisible à l'action de la zymase. 2. Le sel de Ca de l'éther phosphoré se décompose plus lentement que l'éther libre. 3. Le sel de Ca de l'acide pyruvique, si celui-ci vraiment est un produit intermédiaire, ne fermente pas si bien que l'acide libre et en outre, la marche de la réaction étant dérangée, l'aldéhyde acétique apparaît, ce qui concorde aussi avec des expériences de Fernbach et Schoen [1913, 1914]. Les expériences ultérieures montreront quelle des trois explications est la plus probable.

En ce qui concerne l'acide glyoxylique il faut noter ici seulement le fait de la formation de $CH_3.COH$ et par conséquent de $CH_3.CH_2OH$. S'il y a

[1] Ce travail paraîtra bientôt.

vraiment une simple réduction de l'acide, il faudrait admettre qu'il soit un accepteur énergique de H, parce que d'après la réaction

$$CHO.CO_2H + 8H = 2H_2O + CH_3.CH_2OH$$

une molécule de celui-ci pourrait joindre 8 atomes de H ou $\frac{1}{8}$ de son poids. Il est alors possible que la décomposition de $C_2H_2O_3$ soit déterminée par la même cause, puisque dans ce cas une partie des molécules pourrait s'oxyder aux dépens de la réduction de l'autre partie de la même molécule.

L'alcool qui se forme en l'absence de l'air dans les tissus végétaux ne résulte pas toujours de la fermentation du sucre. Sans parler de celui-ci, peut-être faudra-t-il ajouter aux sources connues depuis longtemps comme, par exemple, l'acide pyruvique (Neuberg) et glycérique (Lebedev), encore l'acide glyoxylique que l'on trouve si souvent dans les plantes. L'éclaircissement de cette question se rapportant entièrement au domaine de la physiologie végétale ne faisait pas l'objet de mes recherches.

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# VII. SUR LA FORMATION DES ÉTHERS PHOSPHORÉS PENDANT LA FERMENTATION ALCOOLIQUE.

*(Communication préliminaire.)*

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IL y a quelques années j'ai démontré [1909, 1910, 1911] que pendant la fermentation du sucre l'éther phosphoré d'un hexose se formait. Dans le schéma que j'ai donné j'ai admis que préalablement il se formait un triose-phosphate. Cette supposition a été appuyée par le fait que la dioxyacétone donnait en fermentant un hexose-phosphate identique, évidemment par la condensation de l'éther phosphoré du triose [Lebedev, 1911, 2, 3; 1914; Lebedev et Griaznov, 1912]. En vue de l'importance de cette question, j'ai repris depuis une année une étude minutieuse pour voir si ce point de vue était juste. En modifiant la méthode dont je me suis servi auparavant pour l'isolement de l'éther et ayant pris une autre levure, j'ai pu, après un long et assidu travail, enfin constater d'une manière irréfutable la formation d'un éther ou plutôt d'un mélange des éthers phosphorés qui m'a donné les *p*-bromphénylhydrazone, *p*-bromphénylosazone et phénylosazone ayant un autre point de fusion et une autre composition que les combinaisons correspondantes, données par l'hexose-biphosphate. Les circonstances qui ne dépendent pas de moi m'ont obligé malheureusement d'interrompre pour une demi année l'étude commencée avec tant de succès; c'est pourquoi je me suis résolu de publier le résultat obtenu sans attendre l'accomplissement du travail en train ayant le but de jeter une lumière sur la décomposition primaire du hexose.

*Méthode.* 50 gr. de levure de Lebedev + 100 gr. de saccharose + 35 gr. de $Na_2HPO_4$ + 17 gr. de $NaH_2PO_4$ + 500 gr. d'eau + 1 cc. de toluène. Pour interrompre la fermentation, on ajoutait au moment voulu 2 ltr. d'alcool à 97°. On essorait le résidu à la trompe, on le lavait par de l'alcool dilué (4 : 1) et on le traitait par l'eau à plusieurs reprises; le filtrat (300 cc.) a été précipité par de l'alcool (1200 cc.). Après avoir laissé reposer le tout pendant 12 heures,

on a transvasé l'alcool qui a été au dessus du résidu, on a redissous celui-ci dans l'eau et précipité par 3 volumes d'alcool. (La dernière fois on a précipité par 2 vol. d'alcool.) Par ce procédé on obtient 2 couches de liquide: la supérieure blanchâtre, opaque et très visqueuse, l'inférieure jaunâtre, presque transparente et moins visqueuse. Il était évident qu'il y avait au moins 2 substances différentes. Les deux couches ont été séparées à l'aide d'un entonnoir à robinet et chacune d'elles a été précipitée par l'acétate de plomb. Le résidu a été lavé avec une solution faible d'acétate de plomb et essoré à la trompe. La résidu a été délayé dans l'eau et traité par $H_2S$. Le filtrat a été traité avec de la phénylhydrazine à 100° ou avec une solution alcoolique de *p*-bromphénylhydrazine à 35°. On séchait les précipités après recristallisation dans l'appareil d'Abderhalden, modifié par moi [1911, 1, p. 257] à 37° et on les analysait. On a dosé le P par l'excellente méthode de Neumann.

Dans quelques expériences, avant de traiter avec de l'acétate de plomb, j'enlevais l'acide phosphorique au moyen du mélange magnésien. Sans décrire ici en détails toutes les expériences nombreuses que j'ai faites dans des conditions différentes, je n'en ferai connaître que quelques-unes, pour les autres je donnerai plus bas l'aperçu général des dosages des P et N des osazones recristallisées, obtenues ordinairement par l'action de la phénylhydrazine sur le mélange des deux couches. Il faut les envisager pour le moment comme des expériences d'orientation.

1. La fermentation allait 2 h. à 35°. L'osazone obtenue de la couche supérieure, recristallisée une fois de l'alcool à 85°. P—2·96 %. De grands faisceaux de très longues aiguilles d'une couleur orange. Le point de fusion 142°. L'osazone de la couche inférieure avait le point de fusion 142–144°. P—4.42 %.

2. La durée de la fermentation 4 h. à 25°. L'osazone de la couche supérieure. P—3·96 %. N—20·54 %. L'osazone de la couche inférieure. P—4·21 %.

3. L'osazone de la couche supérieure 6 fois recristallisée. P—1·89 %. Lamelles jaunes. F. 141–142°. Il est à remarquer qu'après le traitement de la solution des éthers phosphorés avec de la phénylhydrazine à 100° on obtient souvent de très longues et larges lamelles jaunes qui après la recristallisation se transforment en aiguilles de couleur jaune ou orange. Il arrive cependant que la forme des lamelles persiste malgré plusieurs recristallisations, néanmoins le point de fusion ne change pas d'une manière sensible, en balançant dans des limites assez étroites autour de 142°, et la couleur reste toujours jaune foncée.

4. Supposant que les éthers phosphorés donnent des sels d'une différente solubilité, j'ai appliqué la précipitation fractionnée par l'acétate de plomb. Pour la première fraction j'ai pris à peu près une moitié de la quantité qui était nécessaire pour faire la précipitation complète des éthers. Le dépôt a été essoré et le filtrat traité de nouveau par l'acétate de plomb. Ainsi a-t-on obtenu une fraction qui traitée par de la phénylhydrazine a donné l'osazone de la composition suivante: N—15·96 et 16·28 (deux dosages); P—4·47; C—54·70 et H—5·63.

Il est curieux de noter ici que ces nombres, à l'exception de celui de C (l'osazone est très difficile à brûler), correspondent presque exactement à la formule:

$$\begin{matrix} O \\ HO \end{matrix} \diagdown\!\!\diagup P \diagup\!\!\diagdown \begin{matrix} O - C_4H_5(OH)_3 - CN_2HC_6H_5 - CHN_2HC_6H_5 \\ O - CH_2 - CN_2HC_6H_5 - CHN_2HC_6H_5 \end{matrix}$$

c'est à dire à l'osazone de l'éther phosphoré composé qui contient pour au moins[1] une molécule de $H_3PO_4$ une molécule de hexose et une molécule de triose.

| | P | N | C | H |
|---|---|---|---|---|
| Calc. pour l'osaz. du hexose-triose-phosph. | 4·51 | 16·28 | 57·56 | 5·38 |
| Trouv. „ „ „ „ | 4·47 | 16·12 | 54·70 | 5·63 |
| Calc. „ „ hexose-biphosphate | 5·68 | 15·38 | 52·75 | 5·68. |

Je ne regarde point cette supposition comme démontrée, mais je ne doute pas que les recherches ultérieures fourniront un très important résultat.

Je donne ici 2 séries de nombres pour P et N des osazones obtenues dans les expériences, dont la température variait de 25 à 35° et la durée de 2 à 5½ h. Après la fermentation pendant 9½ h. on n'a pu obtenir *aucune* osazone. C'est la meilleure preuve que le mélange d'éthers se forme au début de la fermentation et qu'il est fermentescible; d'ailleurs on peut facilement le démontrer par l'expérience directe. Le maximum de la quantité d'éthers se forme à 35° après 2 h. et à 25° après 4–5 h., si l'on a, bien entendu, une levure très active.

Voici un exemple du rapport de la couche supérieure à la couche inférieure du liquide sirupeux. A 35° après 2 h. la couche supérieure contenait 15 cc., la couche inférieure—30 cc. Après 4 h. la couche supérieure—20 cc.; la couche inférieure—25 cc. La couche supérieure est plus soluble dans l'eau que la couche inférieure, de sorte que la quantité de celle-là dépend aussi de la quantité d'alcool employé pour la précipitation. D'autre part ces 2 couches

[1] Sous l'action de la phénylhydrazine une partie de $H_3PO_4$ pourrait se séparer de l'éther phosphoré.

se dissolvent en partie l'une dans l'autre, ce qui empêche extrêmement la séparation par des méthodes purement physiques.

Dans de différentes expériences on a obtenu pour P et N des chiffres que l'on peut diviser en deux groupes.

1. 4·55, 4·37, 4·32, 4·29, 4·82, 4·85, 4·42, 4·21. Moyen: 4·48 % de P.
2. 5·48, 5·39, 5·84, 5·72, 6·01. Moyen: 5·69 % de P.
1. 21·20, 21·14, 19·12, 21·10, 20·54, 18·46. Moyen: 20·26 % de N.
2. 15·96, 16·65, 16·28, 16·82. Moyen: 16·43 % de N.

Le point de fusion variait entre 137° et 150° (toutes les osazones ont été recristallisées au moins une fois). Pour les osazones non recristallisées le point de fusion le plus bas a été 136°. Je rappellerai encore une fois que l'osazone du hexose-biphosphate a le point de fusion 150–152° et contient 5·68 % de P et 15·38 % de N. L'attention est frappée involontairement par le fait qu'on a obtenu dans quelques expériences des osazones qui contenaient 21·20 et 21·14 (deux dosages de la même substance) et 21·10 % de N. Ces nombres ne diffèrent que très peu de celui pour N de la triosazone, à savoir 20·9 %. Néanmoins, l'osazone qui a donné 21·20 et 21·14 de N contenait 5·84 % de P et avait le point de fusion 138–139° (recristallisée 5 fois); celle qui a donné 21·10 % de N contenait 5·31 % de P et avait comme point de fusion 141–142°. De sorte que si l'on se contente, comme on le fait souvent en analysant des osazones, de ne doser que l'azote, sans faire en outre attention au point de fusion, on peut croire qu'on aie à faire avec une triosazone.

Il est à remarquer que c'était justement moi [Lebedev, 1909, p. 123; 1910, p. 223; 1911, 1, p. 256] qui ai obtenu pour la première fois les hydrazones et osazones contenant du phosphore; avant cette découverte on pouvait bien facilement commettre une grave erreur.

Les chiffres pour P, 3·96, 2·96 et 1·89 %, disent en faveur de la supposition que le mélange d'osazones contienne une petite quantité d'osazone qui, ne contenant pas de P et étant moins soluble, s'accumule peu à peu à chaque recristallisation; cependant il est peu probable que ce soit l'osazone du triose, premièrement, à cause de sa grande solubilité dans l'alcool, deuxièmement, par le fait que les chiffres pour N, au lieu de devenir plus grandes, deviennent, au contraire, plus petites. On peut admettre que pendant l'action de la phénylhydrazine en solution acide à 100° une très faible partie du mélange des éthers se décompose en mettant en liberté le sucre qui donne ensuite l'osazone correspondante ou que l'acide phosphorique se sépare partialement du phosphate de l'osazone. J'observais depuis longtemps une chose pareille sous l'action de l'alcali faible sur l'osazone-phosphate; l'osazone qui s'en

séparait avait le point de fusion 215° [Lebedev, 1910, pp. 218–9]. J'ai l'intention de faire plus tard des expériences analogues sur ce sujet.

Quoi qu'il en soit je n'ai jamais obtenu une osazone qui n'aurait pas contenu du phosphore; c'est pourquoi il ne peut y avoir aucune doute qu'Ivanov a fait une erreur [Ivanov, 1907] dans cette question, ce qu'il a d'ailleurs reconnu lui-même [Ivanov, 1915].

Il suit alors de tous les faits rapportés plus haut qu'en disposant les expériences d'une manière convenable on peut isoler un mélange d'éthers phosphorés dont les constituants varient probablement non seulement au point de vue quantitatif, mais aussi qualitatif, selon les conditions, comme, par exemple, la température, la durée de la fermentation, la quantité de sucre ou de phosphate, la réaction du milieu, etc.

Ivanov précipitait l'éther phosphoré après 24 h. par l'acétate de cuivre, mais après un laps de temps si long il pourrait arriver que l'éther avait été fermenté presque entièrement ou même totalement, de sorte qu'il n'y en restait que très peu ou même pas du tout dans le précipité. Ainsi dit-il que l'osazone obtenue après la fermentation du glucose avait au début un point de fusion 142°, mais après quelques recristallisations 127° seulement. Ce fait montre clairement qu'il y avait un mélange qui consistait probablement d'une très petite quantité d'osazone de hexose-biphosphate et d'une très grande quantité d'acétylphénylhydrazine qui se forme, comme on le sait, facilement sous l'action de la phénylhydrazine sur l'acide acétique. Les cristaux ne se purifient que difficilement et ont ordinairement une couleur un peu jaunâtre. Il est évident qu'Ivanov, faisant le dosage de N dans cette substance, a commis une erreur ayant obtenu 20·9 % au lieu de 18·7 %. Une telle erreur est possible si l'on n'est pas suffisamment habitué au dosage de N ou si la substance analysée n'était pas bien purifiée. Le mélange pourrait contenir, outre de l'hexose-phosphate, encore de l'hexose-triose-phosphate et triose-phosphate, sans parler d'autres éthers d'une pareille composition. Cette admission n'a aucun rapport au travail cité d'Ivanov, mais, comme j'ai déjà dit plus haut, a pour point de départ le fait, établi pour la première fois par moi, que la dioxyacétone se transforme pendant la fermentation en hexose-phosphate.

Aussi ai-je commencé des expériences dans lesquelles je me suis servi de la *p*-bromphénylhydrazine; bien que je n'aie fait que 2 expériences, il y a tout lieu à croire que la continuation de celles-ci aura une grande importance pour l'établissement de la formule des éthers, puisque on obtient les hydrazones en forme bien cristalline. La *p*-bromphénylhydrazone se forme en solution

alcoolique après quelques minutes déjà; elle est de la couleur blanche de neige (F. 112–114°), mais après quelque temps, surtout sous l'action de la lumière, elle devient peu à peu jaunâtre, en se transformant évidemment en *p*-bromphénylosazone, ayant le point de fusion 141–142° (jaunes lamelles). Celle-ci, recristallisée une fois, donne de très minces et très longues aiguilles jaunes, entrelacées, qui au microscope ont un aspect de la ouate de verre. F. 143–145°. Je rappelle que les *p*-bromphénylhydrazone et *p*-bromphénylosazone, également obtenues pour la première fois par moi [Lebedev, 1909, 1910, 1911, 1], ont comme point de fusion 128° resp. 165° (non recristallisées). La hydrazone, étant presque insoluble dans l'eau, se dissout facilement dans *N*/2 NaOH avec une jolie couleur rosée. P—6·87 et 6·76 %; calculé pour *p*-bromphénylhydrazone du hexose-biphosphate—7·02 %.

Je n'ai pas encore analysé la *p*-bromphénylosazone. J'ai fait quelques analyses des sels amorphes de Ba, obtenus d'après 2 méthodes.

1. La solution du sel de Ba a été précipité par l'alcool à 97° en proportion 1 : 1; le précipité a été lavé par un mélange d'alcool et d'eau (1 : 1), dissous dans l'eau et précipité de nouveau par un volume égal d'alcool. Ba—41·55 %; P—8·30 %.

2. Le sel de Ba a été précipité par ébullition, filtré, lavé par l'eau bouillante, redissous et précipité de nouveau par ébullition. On a obtenu 40·87 % de Ba. Ba et P, calculés pour hexose-biphosphate, 44·92 % resp. 10·16 %.

Bien que les analyses de toutes les substances décrites plus haut ne soient pas encore complètes, on voit clairement qu'on a à faire avec un mélange au moins de deux éthers phosphorés. Par ce fait le cas devient difficile à résoudre, mais je ne doute pas que j'y réussirai tout de même, étant donné que toutes les substances, à l'exception des sels, sont cristallines et qu'on peut en avoir tant que l'on veut.

La réponse à la question de la décomposition du sucre dépend de la résolution du problème que je me suis posé, indépendamment des travaux de qui que ce soit, depuis si longtemps.

Je me réserve donc de continuer les recherches dans la direction indiquée dans ce mémoire.

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# VIII. THE DIFFERENTIAL BEHAVIOUR OF THE ANTINEURITIC AND ANTISCORBUTIC FACTORS TOWARDS ADSORBENTS.

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(*Received February 7th, 1918.*)

Although we have as yet no definite information about the chemical identity of the antineuritic principle, several of its important properties have been disclosed by the researches of the last decade. Its behaviour towards certain adsorbents is an interesting feature. Chamberlain and Vedder [1911] observed that the antineuritic substance was removed by filtering through bone black. More recently Seidell [1916] has found that a pure preparation of fuller's earth, known as Lloyd's reagent, will also adsorb it and if used in suitable quantity will remove the active principle quantitatively, becoming at the same time activated itself. Williams and Seidell [1916] employed this procedure in order to isolate the active principle from autolysed yeast. They obtained a substance which was identical with adenine and which retained a certain degree of antineuritic activity for some time. Pure adenine from yeast has been shown however by Voegtlin and White [1916] and ourselves [1917] to be inactive. It is therefore probable that the adenine obtained by Williams and Seidell was only associated with the antineuritic factor in yeast in the same way as nicotinic acid has been shown to be associated with it in the extract of rice polishings [Barger and Ewins, quoted by Barger, 1914, p. 112; Drummond and Funk, 1914]. Both when purified become inactive. It is seen therefore that the adsorption procedure has not yet enabled us to isolate the looked for substance.

Very little is known about the chemical and physical nature of the antiscorbutic substance present in many plants and fruit juices, which is capable of preventing and curing scurvy. We know that it is not identical with the antineuritic factor, since substances showing marked antineuritic activity are antiscorbutically inactive and vice versa. There is also evidence that

the antiscorbutic factor is more thermolabile than the antineuritic. It was our original intention to study the behaviour of the antiscorbutic towards adsorbents and in the event of adsorption to study its chemical nature. We have however found that unlike the antineuritic substance it showed under the conditions which have hitherto been employed no tendency to be adsorbed by the materials which removed the former. This differential behaviour of the two active principles towards adsorbents suggested to us the possibility of separating the two when present in the same solution. For want of a natural substance which would contain both in marked quantities we have mixed equal quantities of autolysed yeast and orange juice, the former being antineuritically very active, the latter rich in the antiscorbutic. We then found that we were able by means of fuller's earth quantitatively to remove the antineuritic substance, leaving the antiscorbutic unchanged. The description of the above experiments forms the object of this communication.

## Experimental.

Mention was made above of Seidell's work on the adsorption of the antineuritic factor by fuller's earth and we have taken the opportunity of repeating the experiments. Being unable to procure Lloyd's reagent we made use of ordinary fuller's earth. Brewer's yeast after having the wort pressed out was autolysed at 37° and filtered through a Buchner funnel. The filtrate was then shaken for 15 minutes with fuller's earth (50 gms. of fuller's earth to 1 litre of the autolysed yeast), centrifuged, and the supernatant liquid decanted, this treatment being carried out three times. The resulting autolysed yeast fluid was tested curatively and protectively.

*Exp.* 1. The following experiment describes the action of the autolysed yeast after treatment with the adsorbent on a polyneuritic bird.

Pigeon E showed retracted neck and general paralysis. Received 5 cc. of the treated autolysed yeast. Next day very slight improvement noticeable. On the third day the bird had a relapse and a further 15 cc. was administered. As no improvement was noticeable during the day another 10 cc. was given at 3 P.M. on the same day. The bird was found dead next morning. All the doses were delivered direct into the crop by means of a stomach pump.

The above experiment shows that the autolysed yeast had been entirely inactivated by treatment with fuller's earth.

*Exp.* 2. For the protective experiment a batch of autolysed yeast was divided into two portions. One portion was treated with fuller's earth as described above while the other was left untreated. Four pigeons, *H*, *I*, *K*,

and *L*, were used. All the birds were fed artificially with 80 gms. of rice a day in two portions of 40 gms. each. In addition the birds *H* and *I* received daily by means of the stomach pump 3 cc. of untreated autolysed yeast, while *K* and *L* were dosed with 3 cc. of the treated autolysed yeast. Fig. 1 gives a graphic representation of the changes of weight of the individual birds.

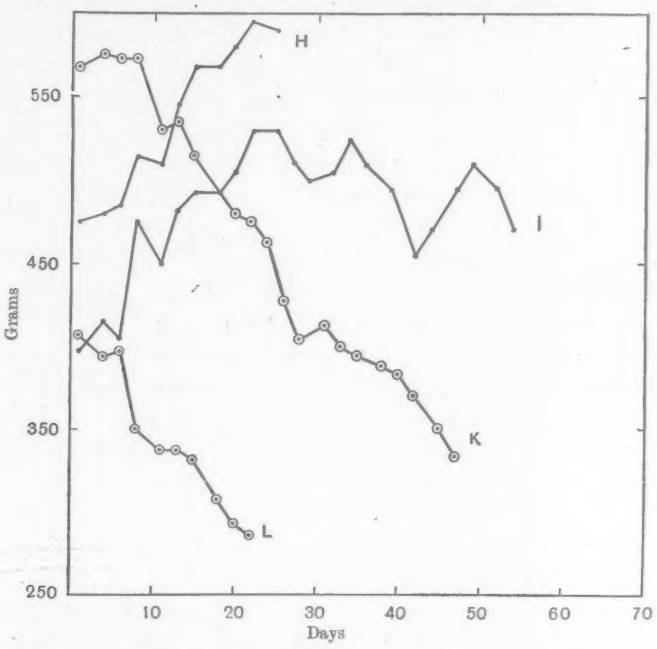


Fig. 1.

It is seen that while *H* and *I* did well on the treatment, even putting on weight, *K* and *L* gradually lost weight, the former succumbing after 46 and the latter after 21 days to polyneuritis. The bird *H* having injured itself had to be killed, while *I* was alive and well at the time the experiment was stopped.

The above experiments fully confirm Seidell's observations.

We have further observed that the active principle is also adsorbed when autolysed yeast is treated with dialysed iron.

*Exp.* 3. Our procedure was as follows. 200 cc. of autolysed yeast was shaken with 200 cc. of dialysed iron, and the precipitated iron compound was filtered and pressed with a hand-press almost to dryness. The mass was then shaken with a litre of 95 % alcohol for 3 hours, the alcoholic extract

filtered and evaporated in vacuo at 45–50° and the residue taken up in 30 cc. of water. 10 cc. of the solution cured a polyneuritic pigeon. The following are the particulars of the case.

Pigeon 188 showed very grave symptoms of polyneuritis, convulsions and retracted neck. 10 cc. of the solution was given per os in the morning; gradual improvement during the day. Next day the bird was almost normal and received another 10 cc. of the extract. The following day the pigeon was quite normal and walked and flew freely. It was then put on a rice diet and developed another attack of polyneuritis seven days after the cure.

We have also observed that when autolysed yeast is treated as described above the filtrate still retains some activity. There is however little doubt that by treating the autolysed yeast with a suitable quantity of dialysed iron the entire content of the antineuritic principle could be removed as in the case of fuller's earth. We have not however as yet pursued the subject any further on these lines.

It has already been mentioned that our adsorption experiments with antiscorbutic solutions were primarily undertaken with the object of isolating or concentrating the active principle. The adsorbents that were able to remove the antineuritic factor from active solutions suggested themselves as suitable substances for the removal of the antiscorbutic. We therefore employed dialysed iron and fuller's earth for the purpose.

*Exps.* 4 *and* 5. 120 cc. of dialysed iron was added to 60 cc. of orange juice, which as is well known is a strong antiscorbutic, and shaken for some time. The precipitated iron was then filtered, pressed almost to dryness and shaken with 95 % alcohol for about 2 hours, the alcoholic extract being then filtered and concentrated in vacuo at a temperature of 30–40°. One third of the extract, which was equivalent to 20 cc. of the original orange juice, was used as a daily dose for an animal. The treatment with the fuller's earth was very similar. 10 g. of fuller's earth was employed for 60 cc. of orange juice, and after being shaken, filtered, and pressed out, was extracted with one litre of 95 % alcohol containing 0·5 % of citric acid for 2 hours. The alcoholic extract was concentrated in vacuo at 30–40° and, as in the case of the dialysed iron, one third of the quantity was used as a daily dose. Although 5 cc. of orange juice is known to be ample as a preventive dose in a guinea-pig we thought it advisable to employ the equivalent of 20 cc. in case the adsorption should turn out to have been incomplete.

We tested the antiscorbutic potency of our extracts on guinea-pigs. Holst and Frölich [1912] in their classical work have shown that if guinea-pigs

are fed on certain diets they develop a condition which is pathologically identical with human scurvy. Oats, bran and water prove to be a very suitable diet for producing experimental scurvy in guinea-pigs and we have accordingly adopted it. In some of our later experiments, in view of the results obtained by Chick and Hume [1917], autoclaved milk was substituted for the water. We found that a daily ration of 50 cc. of autoclaved milk kept the pigs in a better condition but at the same time did not prevent the onset of

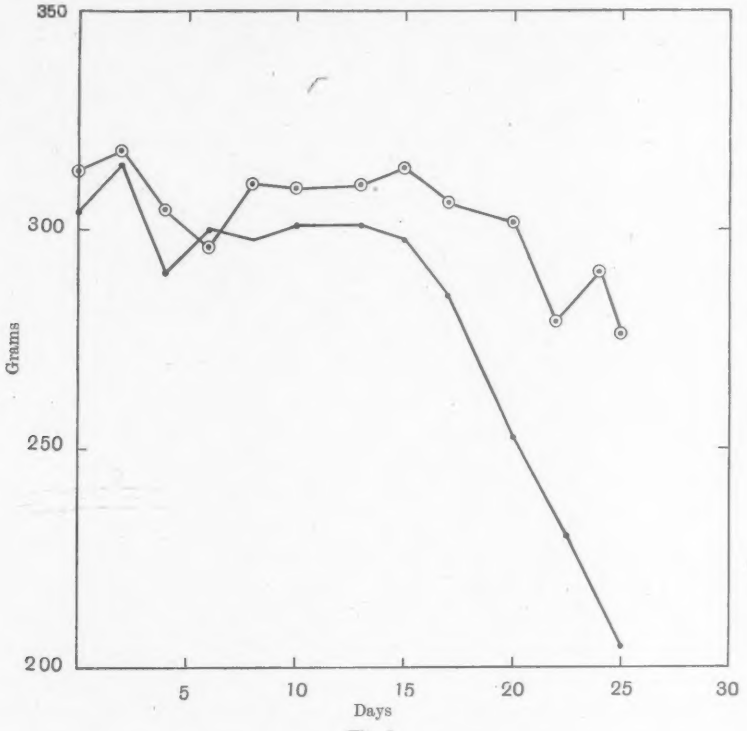


Fig. 2.

scurvy. If guinea-pigs are fed on this diet they commence to drop in weight about 14 to 21 days from the commencement of the experiment and eventually die within a month. At the post mortem a pathological picture very much the same as that of human scurvy is disclosed. Subcutaneous and intramuscular haemorrhages, the latter specially pronounced in the thigh region, are well in evidence. Subperiosteal haemorrhages are also to be observed. The costochondral junctions in the ribs are abnormally enlarged

and haemorrhaged. The bones are very brittle and the teeth are loose. If the scorbutic diet is supplemented with an antiscorbutic, scurvy does not develop and the animal continues its growth in a normal way. For instance, a few cc. of orange juice given in addition to the oats, bran and autoclaved milk will keep a guinea-pig in excellent condition for months.

Our extracts were administered daily per os by means of a syringe, thus insuring quantitative consumption of the dose.

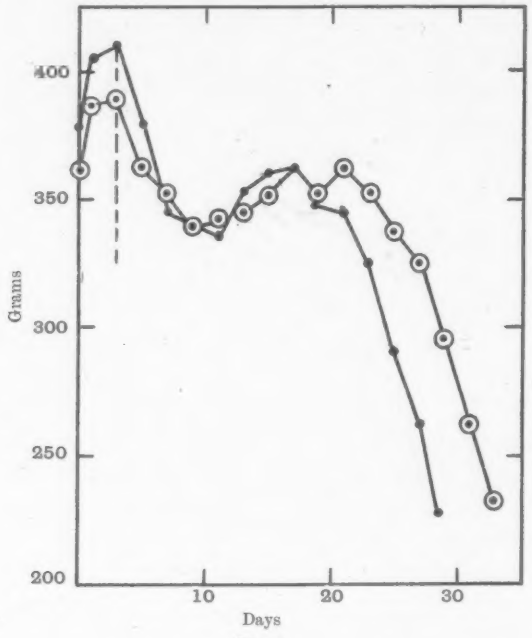


Fig. 3.

The vertical dotted line indicates the date at which the experimental treatment commenced.

Figs. 2 and 3 represent the experiments with dialysed iron and fuller's earth extracts of orange juice.

These curves show the weights of the guinea-pigs employed, two for each experiment. In both cases the animals dropped in weight as they do on a purely scorbutic diet and eventually died within the usual period. At the post mortem they all showed a condition of acute scurvy. The administered extracts neither prevented nor even delayed the onset of scurvy and we are bound to conclude that they were inactive.

The above experiments do not however entirely exclude the possibility of total or partial adsorption of the antiscorbutic by the adsorbents, as the solvents used, *i.e.* 95 % alcohol and acidified alcohol, may not have been suitable for extraction or the adsorption may have been of an irreversible character. To settle this point it was necessary to determine the activity of the solutions after being treated with the dialysed iron and fuller's earth.

For this purpose filtrates of orange juice treated with dialysed iron and fuller's earth respectively, according to the method already described, were tested. The method for the determination of the antiscorbutic potency of a substance is purely biological, and quantitative estimations cannot be carried out with the same degree of accuracy as are chemical estimations. There is very little doubt however that marked differences in the antiscorbutic content of solutions can be ascertained. For instance, we know that in the case of orange juice 3 cc. per day is about the minimum dose [Chick, Hume and Skelton, 1918] that will prevent the onset of scurvy in a guinea-pig and if an extract which is equivalent to 3 cc. of the original juice serves the same purpose we are safe in assuming that the loss of the antiscorbutic potency in the extract, if loss there be, is not very considerable.

*Exps.* 6 *and* 7. It was on these lines that we examined our filtrates. Figs. 4 and 5 show the weight curves of the animals employed.

Guinea-pigs 50 and 52 (Fig. 4) received orange juice treated with dialysed iron. Nos. 65 and 63 (Fig. 5) were dosed with orange juice extracted with fuller's earth, while No. 59 A (Fig. 5) served as a control for 65 and 63, receiving 3 cc. of untreated orange juice. None of the animals developed scurvy, showing that the solutions were active. It is further seen from Fig. 4 that guinea-pig No. 50, receiving an equivalent of 3 cc. of dialysed iron extract, did almost as well as No. 52, receiving 8 cc. As was previously mentioned, 3 cc. of ordinary orange juice is about the minimum preventive dose for a guinea-pig; consequently the antiscorbutic in the juice, if adsorbed at all, could not have been removed by the dialysed iron to any considerable extent.

The experiments with orange juice treated with fuller's earth gave similar results. No. 63, which received 3 cc. of the extracted juice, shows a weight curve almost identical with that of No. 59 A which received the same quantity of untreated orange juice. The juice in both cases emanated from the same batch. No. 65 received 8 cc. of the treated orange juice and developed somewhat better than the others. It must be mentioned here that all these animals were chloroformed at the close of the experiment and at the post mortem examination showed no signs of scurvy.

The above experiments show that the antineuritic and antiscorbutic factors behave differently towards the same adsorbents in their original media. It was of interest to see whether this differential behaviour held also true when the two principles were present in the same medium. We have so far not come across a natural product which contained both in quantities suitable for investigation and we therefore decided to make up a solution which would contain both in ample quantities and test it before and after adsorption.

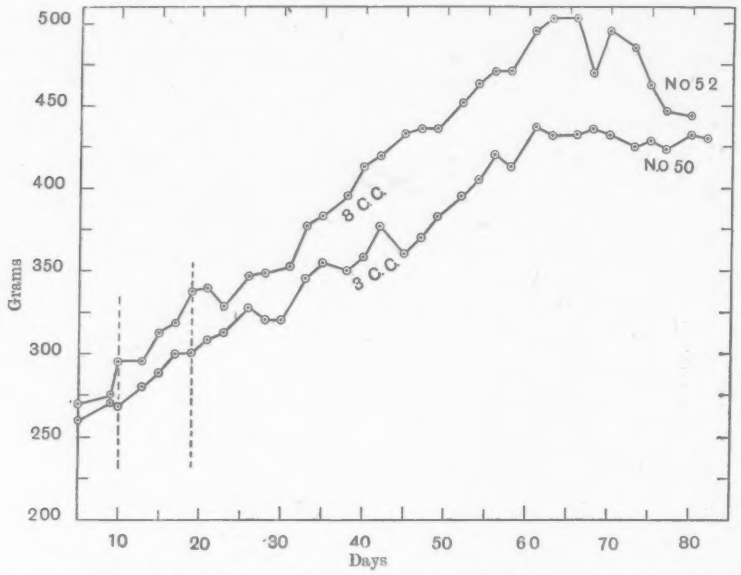


Fig. 4. The first vertical dotted line indicates the date on which the animals were put on a scorbutic diet; the second the date on which the experimental solutions were first given.

*Exp.* 8. A mixture of autolysed yeast and orange juice was therefore employed.

The yeast solution and the orange juice were mixed in equal quantities. One portion of the mixture was treated with fuller's earth three times; the other portion was employed untreated. Each portion was tested curatively and protectively for the antineuritic on pigeons and protectively for the antiscorbutic on guinea-pigs.

As would be expected, the untreated mixture proved effective in curing an acute case of polyneuritis; the mixture treated with the adsorbent however

failed to show any antineuritic potency. The following protocol describes two cases of polyneuritic birds dosed with the treated mixture.

Pigeon $C_2$ showed paralysis and forward head. Received 10 cc. of treated mixture at 4.30 P.M. per os. Next day the bird was worse and another 10 cc. was administered. On the third day the bird was in a much worse condition and died at 2.30 P.M.

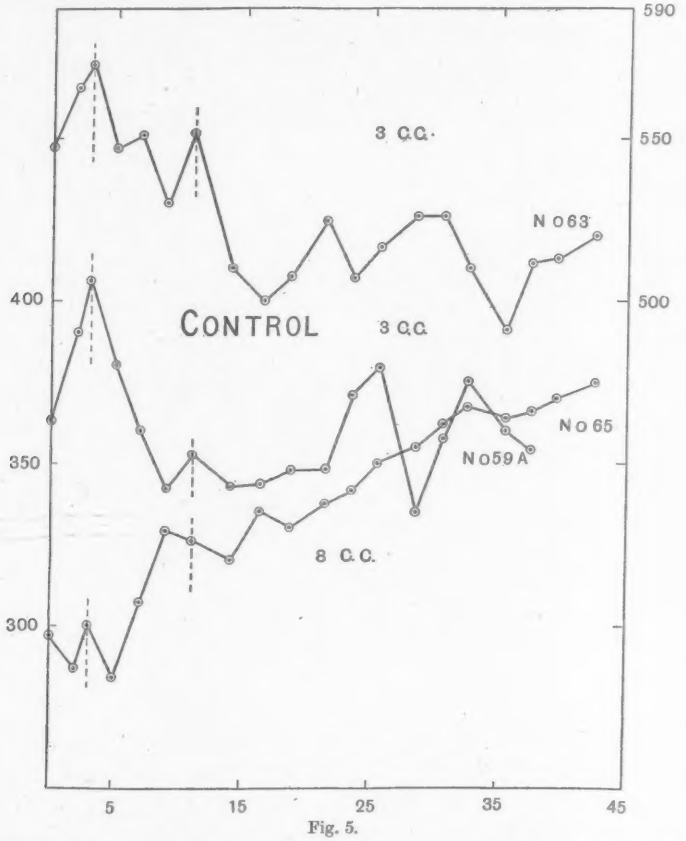


Fig. 5.

Pigeon $D_2$ was found in convulsions showing general paralysis with its head well forward. It received 10 cc. of the treated mixture at 10 A.M. No improvement during the day. Found dead the next morning.

For the protective experiment 10 cc. of the mixture, which was equivalent to 5 cc. of the original autolysed yeast, was administered daily to pigeons fed

on an exclusive diet of polished rice. Four pigeons were employed. Pigeons $A_2$ and $B_2$ received the untreated, while pigeons $A_1$ and $B_1$ were dosed with the treated, mixture. $A_1$ and $B_1$ developed polyneuritis within 33 and 45 days respectively. $A_2$ and $B_2$ on the other hand showed no signs after 50 days. Fig. 6 represents the weight curves of these birds.

It will be seen that the birds in this experiment behaved very similarly to those in the experiment with autolysed yeast alone. Both from the curative and protective experiment it is evident that the treatment with the fuller's earth has inactivated the mixture as it did the unmixed autolysed yeast.

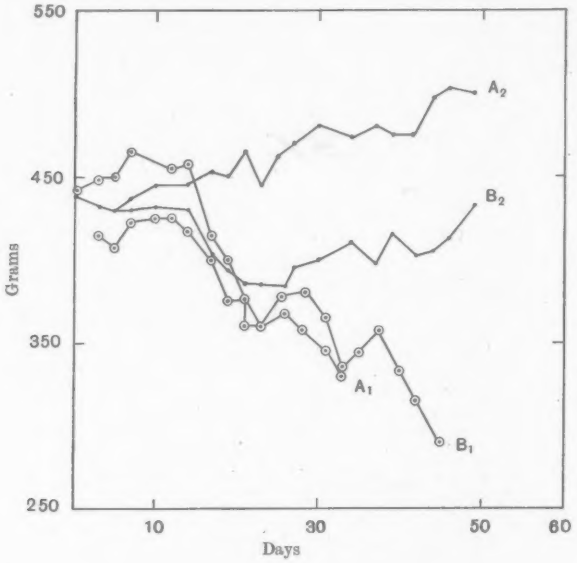


Fig. 6.

To test the antiscorbutic potency of the mixtures four guinea-pigs were employed. One received 6 cc. (equivalent to 3 cc. of orange juice) of the untreated mixture, the other three received 6 cc., 10 cc. and 12 cc. (equivalent to 3 cc., 5 cc., and 6 cc. of orange juice) respectively of the treated mixture. As will be seen from Fig. 7, all the animals developed normally. At the end of two months the guinea-pigs were chloroformed and at the post mortem they were all found in excellent condition without revealing any signs of scurvy. The treatment of the mixture then with fuller's earth has not affected its antiscorbutic potency to an appreciable extent.

We have performed one more set of experiments which had some bearing on the behaviour of the antiscorbutic principle towards adsorbents, namely the effect of filtration of an antiscorbutic through a porous Berkefeld filter. Expressed orange juice contains much suspended matter and is not a solution easily amenable to chemical manipulation. Filtration through filter paper is slow and the resulting filtrate is not very clear. When the juice however is passed through a Berkefeld filter by means of suction a brilliant, clear filtrate is obtained. It was of interest to ascertain whether any of the antiscorbutic had been adsorbed during the process of filtration.

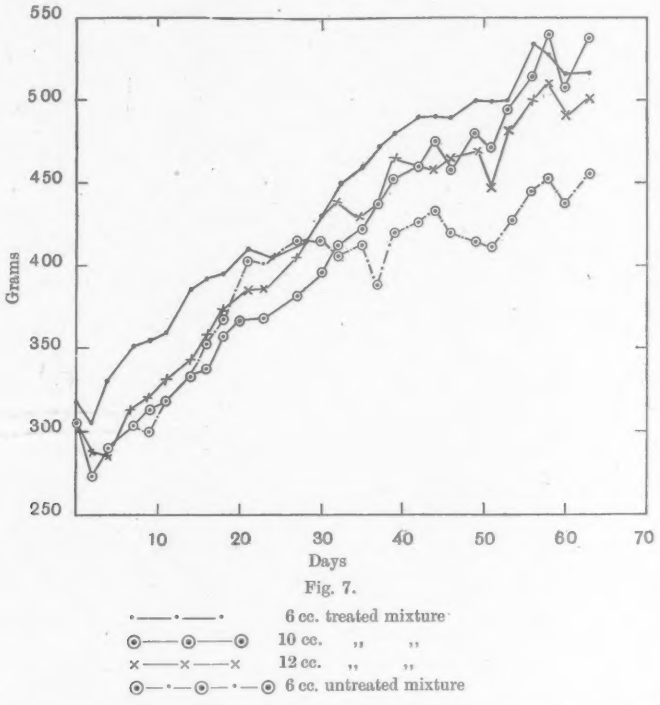


Fig. 7.

·—·—· 6 cc. treated mixture
⊙—⊙—⊙ 10 cc. ,, ,,
×—×—× 12 cc. ,, ,,
⊙—·—⊙—·—⊙ 6 cc. untreated mixture

*Exp.* 9. An experiment was instituted in which three sets of two guinea-pigs each received 3 cc., 5 cc., and 8 cc. of filtered and unfiltered juice respectively. Unfortunately the animal receiving the 3 cc. of the filtered juice succumbed to an unknown complaint 40 days after the experiment commenced. The post mortem did not reveal any signs of scurvy. The animals receiving 5 cc. and 8 cc. of filtered juice failed to develop scurvy and behaved

very much in the same manner as the controls which were given the same quantities of the unfiltered juice. They were all chloroformed and no signs of scurvy were found at the post mortem. Fig. 8 gives a graphic representation of the experiment. These experiments show then that filtration through a porous medium like a Berkefeld filter does not affect the antiscorbutic potency of orange juice.

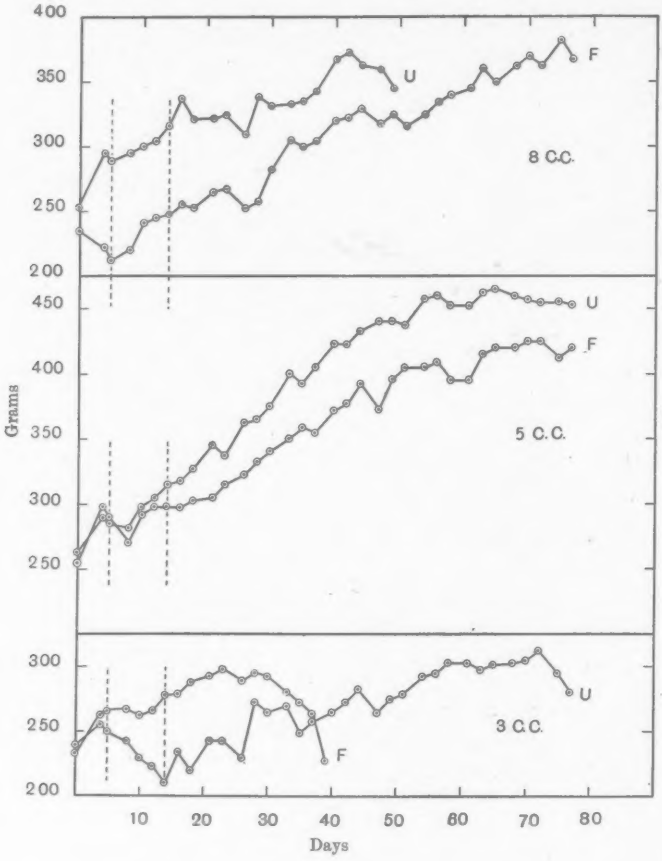


Fig. 8.

U, unfiltered F, filtered

## Conclusions.

The experiments we have described show that under certain conditions at least the antineuritic factor behaves differently towards adsorbents from the antiscorbutic factor; the latter showing no tendency to be adsorbed by substances which remove the former from solution. We have shown that it is possible to separate the antineuritic principle from our artificial mixture containing both factors. Whether this is possible under all conditions we are not at present in a position to say. It is well known that adsorption is regulated by many factors and the H ion concentration of the medium is a condition to be considered. To work this problem out in detail is fraught with many technical difficulties. However it is quite within the limits of our present technique to investigate the behaviour of these accessory factors towards adsorbents in media differing in reaction in a marked degree and we are at present engaged in an investigation on these lines.

## Summary.

1. The authors have confirmed the fact that fuller's earth adsorbs almost quantitatively the antineuritic factor out of autolysed yeast.

2. Dialysed iron also adsorbs the antineuritic factor present in autolysed yeast.

3. Treatment with fuller's earth or dialysed iron does not affect appreciably the antiscorbutic potency of orange juice.

4. On treating a mixture of equal volumes of autolysed yeast and orange juice with fuller's earth the antineuritic factor is removed while the antiscorbutic activity remains unaltered.

5. Orange juice can be filtered through a Berkefeld candle without having its antiscorbutic activity vitiated to any appreciable extent.

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# IX. ON THE NATURE OF THE PROTEOLYTIC ENZYME OF YEAST.

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(*Received December 27th, 1917.*)

The organism of the higher animals possesses a system of ferments hydrolysing the proteins to the stage of their final decomposition—to amino-acids. With regard to two components of this system—pepsin and erepsin [Cohnheim, 1901]—we have sufficient data to allow us to regard them as more simple ferments than animal trypsin. And indeed, pepsin converts proteins into peptones, erepsin acts on peptones, hydrolysing them into amino-acids, whilst trypsin unites in itself both these functions. The latter circumstance leads us to doubt the individual character of trypsin and allows us to regard it as a mixture of two ferments [Cohnheim, 1902; Salkowski, 1902]. However, trypsin cannot be regarded as a mixture of pepsin and erepsin, as, on the one hand, trypsin digests protein more slowly than pepsin, and on the other, it has been definitely shown that trypsin does not act on certain combinations of polypeptides, which are readily acted upon by erepsin.

In invertebrate animals, in which the differentiation of organs stands much lower, it is also much more difficult to observe the specific ferments. Here the hydrolysis of protein to amino-acids is attributed to tryptases [Oppenheimer, 1909, p. 222] which act most efficiently sometimes in an acid, and sometimes in an alkaline medium. Fredericq [1878], for instance, obtained from the tissue of a worm an enzyme which dissolved fibrin better in an acid medium (0·6–1·2 % HCl). We encounter phenomena of a similar kind in investigating the proteolytic ferments in plants. Owing to a weak physiological differentiation in plants it is difficult to solve the question, whether we are here dealing with one ferment hydrolysing the proteins to amino-acids, or whether there is also a system of ferments, which effects the hydrolysis of protein to the end by stages.

From the wide literature on this question I may mention the conclusion arrived at by Hahn [1903], who worked with the juice of yeast obtained by Buchner's method. Hahn thinks that the proteolytic ferment of yeast presents

a new type of digestive ferments, as, similarly to pepsin, the best medium for its action is acid, whilst in its final products of decomposition it corresponds to the tryptic ferment. Hahn named the proteolytic ferment of yeast endotryptase—since it acted inside the yeast cell, and although he was acquainted with the work of Cohnheim on ereptase, Hahn, and other investigators who preceded him, did not express themselves in favour of the complex character of the proteolytic ferments of plants. Butkewitsch [1904, see also Godlewski, 1911], who investigated autolysis of the seeds of *Lupinus angustifolius*, has shown that the process of decomposition of protein is inhibited in an acid medium (0·2 % HCl or 0·1–1 % HCN), and the correlation between the products of decomposition changes altogether, the greater part of them being precipitated by phosphotungstic acid. Evidently, the decomposition of proteins proceeds chiefly to peptones. The supposition that it is possible to alter the correlation between the products of decomposition of protein according to the conditions of the experiment, is corroborated by Fernbach and Hubert [1900]. These authors demonstrated that during the autodigestion of malt the products of decomposition were greatly influenced by the temperature. Thus, at 40° nearly the entire soluble nitrogen belonged to amino-acids, which are not precipitated by phosphotungstic acid. At 60° there were produced only 50–60 % of amino-acid nitrogen, and at 70° its amount did not exceed 40 %, the remaining quantity belonging to the peptones precipitated by phosphotungstic acid. Windisch and Schellhorn [1900] arrived at the same conclusion. The analogy involuntarily comes to mind that in the given case the phenomena taking place are similar to those in the fermentative metamorphosis of starch; and indeed, by heating diastase to 80° it is possible to destroy its function of sugar production, without influencing the dissolution of starch [Pottevin, 1899].

The quantitative alterations of the products of decomposition of protein, observed in different media and at different temperatures, indicate the possible presence in plant tryptase of at least two constituents. The numerous investigations of Vines ("The Proteases of Plants") on the proteolytic ferments of plants have provided experimental data on this question. Vines [1908] regards the protease of plants as an association of "peptase" and "ereptase." The first ferment dissolves the flakes of fibrin, and the second hydrolyses Witte peptone to products giving the tryptophan reaction. This author tried to obtain peptase and ereptase separately in extracts from leaves, bulbs and seeds. Thus, an extract from hemp seeds acidified with acetic acid produced a dense precipitate of protein, which on repeated dissolution in common salt

(NaCl) produced an opalescent solution, which was capable of digesting fibrin, but did not decompose Witte peptone to tryptophan; the filtrate from the protein precipitate contained an active peptonising enzyme (ereptase), but not "peptase." In the next paper [Vines, 1909] this author investigated papaïn in this respect. As regards the ferment of yeast, Vines [1902, 1904, 1905, 1906] discovered that a watery extract of dried yeast digested Witte peptone, but not fibrin, and an extract in 2 % NaCl digested both. The investigations of Vines are very illuminating, but his methods were far from perfect, and the author often sets forth unconvincing experiments for the confirmation of the correctness of his ideas. Thus, he founds all his views on the presence of ereptase in plants on the tryptophan reaction, whereas Butkewitsch [1904] had already shown that coloration by chlorine water does not present a criterion of the deep decomposition of protein, just as the reduction of an alkaline solution of copper oxide, due to the aldehyde group, does not correspond to the complete hydrolysis of the starch molecule to glucose, but appears in the dextrins together with the liberation of their aldehyde group. How careful one has to be in applying the tryptophan reaction for the establishment of the deep decomposition of protein is shown in the experiments of Levene and Rouiller [1907]. These authors decomposed the protein by barium oxide solution and observed coloration by bromine water even after slight hydrolysis; but in the latter case *the substance producing the tryptophan reaction was precipitated wholly by phosphotungstic acid*. As it was impossible to separate tryptophan from such a precipitate, the authors arrived at the conclusion that tryptophan was here in the form of a constituent of a complex compound, probably a polypeptide. Since the polypeptides manifest the tryptophan reaction, it cannot be used for the detection of ereptase at all. Of course, it was also impossible to identify the ereptase with Cohnheim's erepsin, as for this purpose it would have been necessary to show that ereptase is capable of hydrolysing combinations of amino-acids which are inaccessible to trypsin. In general, for the sake of uniformity, Vines' terms ought to be retained and a rational nomenclature of ferments adopted. The ferment splitting the peptones to amino-acids should be named, in agreement with Oppenheimer [1909] *peptase*, without deciding *à priori*, whether it is to be identified with Cohnheim's erepsin, or with one of the constituents of animal trypsin acting on peptones. The ferment which dissolves the protein and converts it into peptone should be named *protease*, using this term in a restricted sense, and not denoting by it the sum of proteolytic ferments. Thus, owing to Vines, we are able to distinguish in plant trypsin at least two constituents, the protease and the

peptase. As regards the protease, which is equivalent in its significance to pepsin, it became known after the fundamental investigations of Hooker [1874] and Darwin [1888] on insectivorous plants. These authors arrived at the conclusion that the secretions of insectivorous plants contain an enzyme similar to the *pepsin* of animals, *i.e.* digesting fibrin and other proteins in an acid medium. After the detailed investigations of Vines [1897, 1898, 1901] on the juice of *Nepenthes*, the experiments of Abderhalden and Terruchi [1906] have shown that this juice, just as in *Drosera*, does not hydrolyse the peptides, *i.e.* is identical with the pepsin of animals. Thus, the presence of pepsin in insectivorous animals points to a high degree of physiological differentiation, since pepsin appears only in the higher animals, whilst in the invertebrate animals it is either not observed at all, or its presence is disputable. The idea of the identity of pepsin in plants and animals (the "pepsin-idea" of Vines [1909]) was the starting-point in the investigation of the proteolytic ferments of plants, and only after Green [1890] had discovered in seeds a ferment hydrolysing proteins to amino-acids could be added to it the "trypsin-idea." At present Abderhalden's method [Abderhalden and Schittenhelm, 1906; Abderhalden and Dammhahn, 1908; Abderhalden and Rilliet, 1908; see also Euler, 1907], applied by himself and his pupils, has assumed great importance in the study of peptase in plants. It is founded on the change in the rotation of the solution of optically active polypeptides after the addition of the ferment. By this method it was possible to demonstrate [Abderhalden and Brahm, 1908] that, if the juice of yeast is diluted in a certain manner, it hydrolyses added polypeptides, similarly to intestinal juice, quite identically and at the same rate; the peptase of yeast could thus be identified with Cohnheim's erepsin.

In my experiments on the proteolytic ferments of yeast I adopted Stutzer's method of estimation of nitrogen in protein. This method of quantitative estimation of nitrogen in the precipitate of copper hydroxide has evoked wide criticism. The most essential arguments were raised by Schjerning [1900], who directed attention to the facts that peptones were not precipitated by copper, and that amides passed into the precipitate. However, in my experiments amides are not encountered in the process of autolysis of yeast and the peptones in the filtrate may be determined by other methods. Stutzer's method is inapplicable only in the presence of a large quantity of carbohydrates, for recently Kostytschev and Brilliant [1916, see also Maillard, 1913] have pointed to a remarkable fact—the union of amino-acids with sugar without any intervention of ferments. This product gave with copper hydroxide a compound insoluble in water. In other cases Stutzer's method gives good

results; it should only be remembered that *from the diminution of nitrogen in the precipitate of copper hydroxide the conversion of proteins can be guaranteed only to peptones*, as they are no longer precipitated by copper. For the estimation of the work of peptase, *i.e.* the conversion of peptones into amino-acids, other methods are required, and I estimated the nitrogen in amino-acids by means of the improved apparatus of van Slyke [Plimmer, 1908]. By combining both these methods it was possible to determine the action of both protease and peptase. My experiments were conducted either with hefanol (a Munich preparation of dried yeast), or with dried yeast (Lebedev). In 1912 I published the results of my experiments on the influence of phosphates upon the proteolytic decomposition during autolysis of yeast. Using different temperatures, adding $K_2HPO_4$ or $KH_2PO_4$ for the production of alkaline and acid media, I estimated in the filtrate—after treatment by copper, according to Stutzer—the amount of nitrogen in the precipitate produced either by lead acetate or phosphotungstic acid. It was found that the acid phosphate ($KH_2PO_4$) had the effect of considerably increasing the decomposition of protein, judging by the precipitate of copper oxide; however, additional precipitation of the filtrates by phosphotungstic acid showed that in proportion to the surplus amount of protein decomposed in the phosphate portion, the surplus of nitrogen in the phosphotungstic precipitate also rose. Thus after 67 hours' autolysis of hefanol at 52–58° the amount of nitrogen in the *normal portion* was:

| | |
|---|---|
| in the copper precipitate ... ... | 145·7 mg. |
| in the phosphotungstic acid precipitate | 131·1 „ |
| Total ... | 276·8 „ |

and in autolysis with 1 % $KH_2PO_4$:

| | |
|---|---|
| in the copper precipitate ... ... | 118·9 mg. |
| in the phosphotungstic acid precipitate | 163·2 „ |
| Total ... | 282·1 „ |

The same results were obtained when lead acetate was employed for precipitation, instead of phosphotungstic acid. Assuming with Vines the presence of two constituents in the proteolytic ferment of yeast, I had already at that time arrived at the conclusion that *$KH_2PO_4$ increases the decomposition of protein only to the stage of the high-molecular products of decomposition of protein—peptones*—which, as is known, are precipitate dboth by phosphotungstic acid and lead acetate. Therefore, in the case quoted we can speak only of stimulation of protease[1].

[1] In my work of 1912 I used Vines' terms "peptase" and "ereptase," which must now no longer be employed.

In order to ascertain this point finally I have estimated the quantity of amino-groups by van Slyke's method in the normal and phosphate portions.

*Exp.* 1. Four portions of hefanol of 1·1 g. were set for autolysis with 25 cc. of water at 46°. To 2 and 4 were added 0·37 g. $KH_2PO_4$ in each and to all toluene. The experiment was conducted for 43 hours. The initial quantity of protein-nitrogen in the portions varied between 83·4 mg. and 84·2 mg.; the amounts of nitrogen obtained are, for the sake of convenience, re-calculated to portions containing 100 mg. of protein-nitrogen. In 1–2 the nitrogen was estimated according to Stutzer, in 3–4 according to van Slyke.

| Nos. of portion | Composition of portion | Decomposed protein in mg. of protein-nitrogen | Newly formed amino-acid nitrogen |
|---|---|---|---|
| 1 and 3 | Water | 37·8 | 14·6 |
| 2 and 4 | 1·5 % $KH_2PO_4$ | 51·8 | 14·9 |

Here, notwithstanding the greatly increased amount of protein decomposed in the presence of acid phosphate, the quantity of nitrogen according to van Slyke, *i.e.* the nitrogen of amino-acids, did not increase. It appears that *the given conditions have created surroundings favourable for protease, but these conditions (acid medium) have in no way accelerated the work of peptase.*

Saleski and Schatalov [1915], who have revised my previous experiments with phosphates, have arrived at the same results, but they could not explain the fact that in my experiments the difference between the normal and phosphate portions did not disappear with time, and at the end of autolysis remained the same as after 21 hours. Indeed, after 46 hours of autolysis at 54–58°, the decomposition of protein in my experiments was in the normal—58·5 %; phosphate—73·5 %, and after 72 hours—normal—56·9 %; phosphate—73·1 %. In all cases, when autolysis was conducted at a higher temperature, rising to 58°, this difference was maintained, but at a temperature of 34° the difference between the normal and phosphate portions disappeared after 7 days, and at 18° autolysis during 10 days gave:

normal—69·6 % of protein-nitrogen,
phosphate—68·4 % of protein-nitrogen.

This phenomenon is explained by the fact that *at high temperature the work of peptase is at first interrupted, and later altogether suppressed.* The high-molecular product of protein decomposition accumulated in these conditions is not hydrolysed further; that is why this difference is maintained.

*Exp.* 2. Portions of about 1 g. dried yeast (Lebedev) with 10 cc. water were set for autolysis with toluene. Amount of protein-nitrogen—100 mg. Temperatures—34° and 54°.

| Nos. | Conditions of experiment | T | Amount of amino-acid nitrogen in mg. in portion | Amount of amino-acid nitrogen in mg. newly formed |
|---|---|---|---|---|
| 1 | Autolysis 90 hours ... ... ... ... ... | 34° | 54·9[1] | — |
| 2 | Same, added 0·2 cc. 10 % NaOH and 20 hours longer | 34° | 55·0 | — |
| 3 | 90 hours, added 0·25 g. peptone and 20 hours longer | 34° | 63·2 | 8·3 |
| 4 | 90 hours, added 0·25 g. peptone + 0·2 cc. 10 % NaOH, and 20 hours longer ... ... ... | 34° | 66·9 | 12·0 |
| 5 | Same as 1, but higher temperature ... ... ... | 54° | 39·8[1] | — |
| 6 | Same as 3, but higher temperature ... ... ... | 54° | 41·2 | 1·4 |
| 7 | Same as 4, but higher temperature ... ... ... | 54° | 42·2 | 2·4 |

[1] To each of the control portions 1 and 5 was added 0·25 g. peptone previously to nitrogen estimation by van Slyke's method.

It follows clearly from the experiment that during 90 hours at 34° the peptase is not destroyed, as the peptone added is hydrolysed by it; this is seen from portions 3 and 4, in which an increase in the nitrogen, according to van Slyke, is observed, especially in the presence of a small amount of alkali. Autolysis at 54° has interrupted the work of peptase, and later has altogether inactivated it, as the peptone added was hardly decomposed, and there was no increase of amino-acid nitrogen, disregarding the 1·4 and 2·4 mg., which are due to a certain decomposition of peptone in water solution at the temperature of 54°, as control experiments have shown.

That acids and acid salts promote the work of the proteolytic ferment has been known since the time of the first investigations of Gorup-Besanez, but from the experiments with acid phosphate we observe that only the work of protease is increased, *i.e.* the conversion of proteins to the high-molecular products of decomposition. In animal physiology it is assumed that under the influence of hydrochloric acid the proteins are converted into acid albumin soluble in water and in this form become easily acted upon by pepsin. The data of Neumeister [1890] prove that before being digested by pepsin the coagulated protein is simply dissolved without any traces of denaturation. The addition of $KH_2PO_4$ in my previous experiments also evoked the dissolution of coagulated protein in the presence of the ferment. Thus, a watery extract of hefanol containing a large amount of heat-coagulable protein was submitted to autolysis with and without $KH_2PO_4$, at temperatures of 32° and 54°. At 32° the experimental portions were quite transparent and the difference in the decomposition of protein was insignificant (54·3 % control and 64·5 % in phosphate). At 54° the control produced 38·5 % decomposition and *contained a precipitate of coagulated protein*, whilst the phosphate was transparent and the decomposition of protein in it was 65·1 %. The presence of

$KH_2PO_4$, probably, evoked the formation of acid albumin and thus accelerated the work of protease; the importance of the acid phosphate was considerable at 54°, when coagulation of the protein took place. Besides this rôle, $KH_2PO_4$ could also serve to convert the pro-enzyme stage of protease into the active form, similarly to the manner in which the pro-pepsin (pepsinogen) of animals is transformed to pepsin by acids. My previous experiments have proved with full evidence that during autolysis at temperatures of 19° and 54° the final products of decomposition of protein will be different. At these lower temperatures hardly any intermediate high-molecular products of decomposition of protein[1] are observed; if the filtrate from the copper treatment is precipitated with lead acetate, the precipitates obtained in the portion autolysed at 19° only one-fifth the size of those obtained at 54°. That shows that at a high temperature the work of peptase is interrupted; this is corroborated by *Experiment* 3. After standing with toluene at 34° peptone does not increase the amino-acid nitrogen but at 54° this is observed to a small degree.

*Exp.* 3. Two portions of dried yeast (Lebedev) to the amount of 1 g. were set for autolysis with 25 cc. water. Toluene added; duration 94 hours. Temperatures 34° and 58°.

| *T.* | Protein decomposed in mg. | Newly formed amino-acid nitrogen |
|---|---|---|
| 34° | 68·8 | 42·6 |
| 58° | 50·4 | 20·9 |

Therefore, *to each* 100 *mg. of protein decomposed at* 34° *correspond* 61·8 *mg. amino-acid nitrogen*, and to the same amount decomposed *at* 58° *there correspond only* 41·5 *mg.* Since it is possible to estimate the action of peptase by the amount of amino-acid nitrogen, it is clear that high temperature inhibits its work.

In my preceding work I reduced the proteolytic ferment of yeast to an inactive state by heating to 80° in water. Hefanol heated in this manner did not produce decomposition of protein during further autolysis, but if acid phosphate (1·5 %) was added to the experimental portion, the decomposition of protein was again observed. In one experiment out of 106·4 mg. of protein-nitrogen, 5·9 mg. were decomposed; in another, in which the temperature was raised to 75°—even 21·3 mg. This decomposition may be ascribed either to the regeneration of the ferment caused by the acid salt, or, which I think more probable, to the conversion of the pro-enzyme under these conditions into the

[1] This is also mentioned by Hahn [1903], who found on autolysis traces of albumoses and a total absence of peptones.

active form. The pro-enzymes are in general more resistant to heating than the ferments themselves. In order to demonstrate that the decomposition of protein takes place here owing to the action of the ferment, and is not due to the influence of the acid phosphate, I carried out experiments with boiled portions and portions inhibited with formalin.

*Exp.* 4. Portions of hefanol of 1·1 g. set for autolysis at 46°, toluene added; 1–2 were treated with 25 cc. boiling water and boiled during 1 minute; to 3–4 were added 25 cc. 1·5 % formalin. Into each of the portions 2 (after cooling) and 4 was introduced 0·37 g. $KH_2PO_4$. Autolysis during 42 hours.

| Nos. of portions | Condition of experiment | Amount of protein-nitrogen *previously to experiment* | Amount of protein-nitrogen *after experiment* |
|---|---|---|---|
| 1 | Boiled ... ... ... | 84·2 | 84·4 |
| 2 | Boiled + $KH_2PO_4$ ... ... | 83·8 | 83·9 |
| 3 | 1·5 % formalin ... ... | 84·9 | 84·1 |
| 4 | 1·5 % formalin + $KH_2PO_4$ ... | 84·4 | 84·5 |

In the result no traces of decomposition of protein were observed even in the phosphate portions after the ferment was inactivated either by boiling or by formalin. What were the products of decomposition of protein obtained after the resumption of the activity of the proteolytic enzyme under the influence of $KH_2PO_4$ after heating at 75–80°? The question was, whether there took place a transition from the pro-enzyme state of protease and peptase, or only from the former. In my preceding work it was observed that in proportion as the decomposition of protein with phosphate was increased, the greater was the amount of nitrogen appearing in the precipitate with lead acetate, as compared with the control portion. I have arrived at the conclusion that in the given case one may only speak of the work of protease carrying out the decomposition of protein to the high-molecular products of digestion. That heating to 75° destroys peptase completely is seen from the following experiment.

*Exp.* 5. Portions of dried yeast (Lebedev) of 1 g. were heated 1·75 minutes to 75° on a water-bath with 25 cc. water. To each of 2 and 3 was added 0·25 g. peptone.

1. Control, nitrogen estimated according to van Slyke ... ... 13·0 mg.
2. Same, but previously to estimation 0·25 g. peptone added ... 15·7 „
3. To portion heated to 75° added 0·25 g. peptone, and left for 20 hours at 56°. ... ... ... ... ... ... 16·0 „

Notwithstanding the 20 hours' digestion at 56°, the amount of amino-acid nitrogen did not increase, as compared with control portion 2. There-

fore, heating to 75° destroys peptase, and only the protease is regenerated in the given conditions under the influence of $KH_2PO_4$.

This is seen from the following.

*Exp.* 6. Four portions of hefanol of 1·1 g. each with 25 cc. water were previously heated on the water-bath for 1·5 min. to 75°; then after cooling, to each of 2 and 4 were added 0·37 g. $KH_2PO_4$ and these allowed to remain 44 hours at 46°. In all toluene. Amount of protein nitrogen estimated to 100.

| Nos. of portions | Nature of portion | Nitrogen in precipitate of $Cu(OH)_2$ | Protein decomposed, in mg. of N | Newly formed amino-acid nitrogen |
|---|---|---|---|---|
| 1 and 3 | Control | 100 | 0 | 0 |
| 2 and 4 | Phosphate | 89·6 | 10·4 | 0·8 |

Only the work of protease is present; decomposition was conducted only to the high-molecular products not precipitated by $Cu(OH)_2$, but precipitated by lead acetate.

*Exp.* 7. Same as preceding, but the portions were precipitated with lead acetate after autolysis.

| Nature of portion | N in mg. before experiment | Nitrogen in lead acetate precipitate |
|---|---|---|
| Control | 70·1 | 69·7 |
| Phosphate | 72·0 | 71·9 |

No difference was obtained between the control and phosphate portions, as the high-molecular products of decomposition, protein-albumoses and peptones, produced in the latter case, were precipitated by lead acetate.

In the experiments described above the same phenomenon was repeated as in the autolysis of yeast, in which the ferment was not inactivated by heating; in both cases in the phosphate portions was observed an accumulation of surplus high-molecular products of decomposition, but the quantity of amino-acids did not increase.

Involuntarily there come to mind the analogous results with the fermentative conversion of starch into glucose. I have already mentioned experiments in which by heating to 80° it was possible to inactivate the sugar-forming function of diastase without hindering the solution of starch and its conversion into dextrins. Evidently, in my experiments a complete inactivation of peptase also takes place. My data can be placed in parallel with the experiments on papaïn. Papaïn is regarded as a ferment of the tryptic type and Vines even discovered in it both constituents of the proteolytic enzyme. However, a series of authors [Delezenne, 1906; Jonescu, 1906; Sachs, 1907] had shown that papaïn digested egg-white best at 80–90°; *decomposition proceeds rapidly, but is carried on only to albumoses and peptones.*

While I dealt only with killed yeast—hefanol and zymin—I invariably obtained activation of the protease of yeast by acid phosphate, only sometimes in portions to which phosphate had not been added there was also observed a slight decomposition, probably due to incomplete inactivation of the ferment by heating. But when I carried out similar experiments with dried yeast (Lebedev), it occurred that *rapid* heating to 80° inactivated the ferment altogether, and after gradual heating during 2 min. to 75° decomposition of protein was observed both in the phosphate and control portions, but there was produced no increase of the amino-nitrogen in this decomposition.

*Exp.* 8. Portions of dried yeast (Lebedev) of about 1 g. with 25 cc. water, heated for 2 min. to 75°[1]. Autolysis with toluene 20 hours at 45°. To each of 3 and 4 was added 0·37 g. $KH_2PO_4$ after cooling.

| Nos. of portions | Nature of portion | Decomposition of protein in mg. of nitrogen | Newly formed amino-acid nitrogen |
|---|---|---|---|
| 1 and 2 | Watery | 14·0 | 0 |
| 3 and 4 | Phosphate | 16·6 | 0 |

It is seen that the addition of phosphate hardly produced any effect; this is a general phenomenon in working with dried yeast (Lebedev), as even during autolysis of this yeast with the ferment *not inactivated by heating*, no increase of the decomposition of protein in the presence of $KH_2PO_4$ was observed. Probably, in the given case, the yeast possessed highly active enzymes, and, therefore, the addition of $KH_2PO_4$, which in its essence serves as an activator, does not alter its work. It must also be noted that in Experiment 8 the product of decomposition of protein is nearly wholly precipitated by phosphotungstic acid; thus the amount of protein decomposed, expressed in mg. of protein-nitrogen, was 14·0, and in the phosphotungstic precipitate following copper, 11·9 mg. of nitrogen. This fact confirms the assumption that here are produced the primary products of decomposition of protein, the so-called albumoses and peptones. These primary products of decomposition are obtained in large quantities during autolysis of short duration; thus, during 3 hours' autolysis of dried yeast (Lebedev) at 45° in my experiment, 32·1 mg. of protein were decomposed, and only 2·1 mg. of amino-acid nitrogen were newly formed; or to 100 mg. correspond only 6·2 mg.

[1] It was proved by control experiments that during the 2 min. heating no visible decomposition of protein took place.

Besides high temperature, with the view of depressing the work of peptase I have conducted experiments on autolysis of dried yeast (Lebedev) in the presence of alcohol.

*Exp.* 9. Portions of dried yeast (Lebedev) of about 1 g. with 20 cc. water + 5 cc. 95 % alcohol. Autolysis during 20 hours at 45°.

Amount of protein decomposed in mg. of N 18·2.

Newly formed amino-acid N ... ... 0.

Thus, 19 % alcohol destroys the work of peptase similarly to heating.

In all my experiments which were carried out either in a natural slightly acid medium, or acidified, owing to the presence of $KH_2PO_4$, I have not observed any stimulation of peptase; on the contrary, after a certain time, especially at high temperature, it was altogether inactivated. In the work of 1914 [Ivanov, 1914] I changed the acid medium of the experimental portion to alkaline during the autolysis of yeast, and allowed the decomposition of protein to proceed. I expected that in the alkaline medium, which is most favourable to animal trypsin and peptase [see experiments of Abderhalden and Terruchi, 1906], the decomposition of the intermediate compounds to amino-acids would proceed better; as a matter of fact I observed, on such a change of medium, accumulation of nitrogen determinable by Stutzer's method, and a corresponding diminution of nitrogen in the lead acetate precipitate, in which the presence of peptones could have been suspected. I at that time arrived at the conclusion that in the given conditions there was a possibility of fermentative synthesis from the products of primary decomposition of proteins. However, the question proved to be more complicated; if the medium became weakly alkaline from a slight addition of secondary phosphate ($K_2HPO_4$), then, as I have previously shown [Ivanov, 1916], in an alkaline medium at the cessation of the decomposition of protein increased formation of amino-nitrogen was observed, *i.e.* the work of peptase. Thus, in one experiment after complete cessation of the decomposition of protein, the amount of amino-acid nitrogen rose from 78·8 mg. to 102·2 mg. after an addition of alkali and renewed autolysis during 24 hours. At that time I concluded that toward the end of autolysis "there is accumulated a certain amount of polypeptides not precipitated by copper, and with difficulty acted upon by ferments; the addition of alkali and high temperature evoke a retardation in the decomposition of protein, but facilitate the decomposition of these polypeptides."

Thus, it was possible to separate the two constituents of the proteolytic ferment by function; *in this case, in the alkaline medium the protease was inactive, while the peptase acted*; and in the experiments described above the

peptase was inactivated by heating to 75° and only the protease manifested activity in the presence of acid phosphate.

In the above-mentioned work of 1916 I succeeded in establishing the presence of phenylalanine amongst the products of decomposition of protein in alkaline medium. E. Fischer and Abderhalden [1903] consider that during digestion of protein by animal trypsin, phenylalanine (and proline) never appear amongst the products of decomposition, on account of the polypeptides which contain them being resistant to the action of this ferment. And since the hydrolysis of these polypeptides is possible only by erepsin, the peptase of yeast may be regarded as identical with the erepsin of animals.

After the conclusion of the present investigation, I received the recently issued excellent paper by K. Dernby [1917]. The author applied exact methods for the determination of the concentration of the H-ions of the medium and demonstrated that there exist corresponding optima of the concentration of H-ions for pepsin, trypsin and ereptase of yeast. Thus, according to Dernby, the proteolytic ferment of yeast does not consist of two constituents —protease ("pepsin") and ereptase, as might be supposed, on account of previous experiments, but of three; to them is added also tryptase, which is analogous in action to the trypsin of animals.

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# X. THE ACETONE BODIES OF THE BLOOD IN DIABETES.

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The analyses of blood given below were made some years ago, but were not published as the method of estimation of aceto-acetic acid employed was found to be unsatisfactory. When known amounts of sodium aceto-acetate are added to urine, a yield of from 97 to 100 % of the theoretical amount of acetone can be obtained by the method published previously [Kennaway, 1914, 1]. But when the same method is applied to blood, a yield of no more than 88 % is obtained, and no means have been found of overcoming this defect. $\beta$-Hydroxybutyric acid shows no such difference in behaviour in blood and in urine; possibly the greater chemical activity of aceto-acetic acid, which is indicated by its constitution [Hurtley and Trevan, 1916], causes it to combine with some constituent of the blood which is not present in urine.

However, no results have been found in the literature which have been proved to be more accurate than those here brought forward; the analytical methods employed previously have been tested not with aceto-acetic acid, but with acetone, and the recovery of acetone from blood is a comparatively easy matter. Moreover, in a recent paper Hurtley [1916] has drawn attention to the great lack of any information on the amounts of acetone bodies present in the blood in diabetes; the analyses of Geelmuyden and of Marriot appear to be the only ones to be found in the literature. Geelmuyden [1909] estimated the acetone bodies in the blood obtained post-mortem from two cases of diabetic coma; in his earlier series [1904] the $\beta$-hydroxybutyric acid was not estimated. Sassa [1914] estimated the $\beta$-hydroxybutyric acid alone in the organs of three diabetics, but did not examine the blood. Marriot [1914] gives the amounts of acetone bodies obtained by his nephelometric method in the blood, withdrawn during life, from eight cases of diabetes; only one of these showed any signs of impending coma. The results from this case, together with those of Geelmuyden, are appended to Table II below for comparison.

### Method of Estimation.

The estimations were made by the combination of the Schaffer and Scott-Wilson method described previously [Kennaway, 1914, 1] for the estimation of acetone bodies in urine. As a rule the addition of basic lead acetate solution (U.S.P.) and ammonia (sp. gr. 0·88) in the proportion of 60 cc. of the former and 25 cc. of the latter for 50 cc. blood produces a perfectly clear colourless filtrate; if too little ammonia be added the filtrate is coloured with reduced alkaline haematin and froths on distillation. The first distillates are redistilled with caustic soda and hydrogen peroxide as described by Schaffer [1908]. This method gives satisfactory agreement between duplicate analyses; the final Scott-Wilson estimations are carried out invariably in duplicate, as ample material is available at this stage.

### Tests of Method.

#### (*a*) *With sodium β-hydroxybutyrate.*

The salt used gave practically theoretical figures on combustion [see Kennaway, 1914, 1]. The loss in the estimations on blood given below is no greater than that observed in estimations on pure solutions of the substance (*i.e.* yield of acetone about 95 % of that calculated [Schaffer and Marriot, 1913; Kennaway, 1914, 1]).

(1) 50 cc. ox blood[1] + 0·1616 g. sodium β-hydroxybutyrate = 74·4 mg. acetone
found 70·3 mg. = 94·5 %.

(2) 50 cc. ox blood + 0·1519 g. sodium β-hydroxybutyrate = 69·9 mg. acetone
found 67·3 mg. = 96·3 %.

#### (*b*) *With sodium aceto-acetate.*

Aceto-acetic ester was saponified in the cold with the theoretical amount of caustic soda, the solution diluted, and the aceto-acetic acid estimated by Scott-Wilson's method.

| | mg. acetone in 25 cc. distillate |
|---|---|
| Calculated from weight of ester | 0·8490 |
| Found ... ... ... ... | 0·8485 |

When such a solution is added to urine a practically quantitative yield of acetone can be obtained, whereas in the presence of blood no more than about 88 % of this amount was recovered by the same method. This is not due to any products of decomposition in the blood used, since freshly drawn blood

[1] The amounts of acetone obtained from the blood alone in a blank estimation were of course subtracted from the result in all these experiments.

(rabbit) does not constantly give better results, although in one such experiment 93·7 % was obtained. Serum causes as much loss as does the whole blood. No relation was found between the percentage lost and the absolute amount present. The following are examples from among a considerable number of experiments.

(1) 50 cc. ox blood + sodium aceto-acetate solution = 70·7 mg. acetone
found 63·1 mg. = 89·1 %.

(2) 50 cc. ox blood + sodium aceto-acetate solution = 30·08 mg. acetone
found 26·07 mg. = 86·7 %.

(3) 50 cc. ox blood + sodium aceto-acetate solution = 30·94 mg. acetone
found 27·16 mg. = 87·8 %.

(4) Sodium aceto-acetate solution treated in same way with basic lead acetate and ammonia, but without blood
calculated 31·20 mg. acetone
found 31·22 mg. = 100 %.

*(c) With acetone.*

20 cc. acetone solution titrated by iodoform method 31·3 mg. acetone
20 cc. added to 50 cc. ox blood 30·5 mg. = 97·4 %.

*Control analyses of non-diabetic bloods.*

These results (Table I) serve merely as controls upon the much larger amounts of acetone bodies obtained in fatal cases of diabetes; they are not to be regarded as showing the actual amount of acetone bodies present in normal blood. It is probable that a large part of the substance obtainable from normal blood which is precipitated by Scott-Wilson's reagent is not acetone at all, for the ratio between the amounts resulting from the operations for the estimation of aceto-acetic acid and $\beta$-hydroxybutyric acid respectively is very irregular in the series. For this reason the total amounts only are given in the Table.

TABLE I. *Control analyses of non-diabetic bloods.*

| | | Total acetone bodies as mg. aceto-acetic acid in 100 cc. blood |
|---|---|---|
| Human cases, at autopsy | Heart failure | 16·8 |
| | Head injury; death 3 hours later | 17·3 |
| | Head injury; death 1 hour later | 6·6 |
| | Head injury; death 1 hour later | 7·6 |
| | Septicaemia | 11·3 |
| | Carcinoma of liver | 4·7 |
| | Eclampsia | 3·0 |
| Sheep | | 5·0 |
| Sheep | | 8·7 |
| Horse (in life) | | 1·7 |
| Ox | | 6·2 |
| Ox | | 4·3 |
| Ox | | 4·5 |

TABLE II. *Amounts of acetone bodies are expressed as mg. of aceto-acetic acid in 100 cc.*

| | Urine | | | | Blood | | | | $NaHCO_3$ given by mouth on last days of life | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acetone + aceto-acetic acid | β-Hydroxy-butyric acid | Total acetone bodies | β-ratio | Acetone + aceto-acetic acid | β-Hydroxy-butyric acid | Total acetone bodies | β-ratio | grams per day | days | Remarks |
| Case 5 | 177 | 306 | 483 | 63 | 109 | 170 | 279 | 61 | 81 | 1 | 7. vii. 13, not in coma; alveolar $CO_2$ 17·6 mm. 8. vii. 13, in coma; alveolar $CO_2$ 8·8 mm. 9. vii. 13, death in coma |
| ,, 6 | 114 | 396 | 510 | 78 | 53 | 132 | 185 | 71 | 39 | 3 | Coma; boy, aged 11 |
| ,, 7 | 149 | 522 | 671 | 78 | 86 | 165 | 251 | 66 | 31 | 3 | 10. iii. 13, not in coma; alveolar $CO_2$ 13·8 mm. 12. iii. 13, coma. 13. iii. 13, death in coma |
| ,, 9 (1) | 370 | 880 | 1250 | 70 | 91 | 152 | 243 | 62 | 70 | 5 | Blood and urine (1) obtained on 30. i. 14; alveolar $CO_2$ then 17·8 mm. Conscious till death on 31. i. 14. |
| ,, 9 (2) | 185 | 422 | 607 | 69 | 96 | 117 | 213 | 55 | | | Blood and urine (2) at autopsy |
| ,, 10 | 229 | 528 | 757 | 70 | 106 | 181 | 287 | 63 | 62 | 1 | 6. i. 14, not in coma; alveolar $CO_2$ 15·0 mm. 7. i. 14, 5 p.m. in coma; alveolar $CO_2$ 7·2 mm. Death at 10 p.m. |
| ,, 11 | 311 | 621 | 932 | 67 | 98 | 169 | 267 | 63 | ? | — | Coma. |
| ,, 12 | 98 | 192 | 290 | 66 | 83 | 125 | 208 | 60 | 40 | 2 | Coma |
| ,, 26 | 628 | 1682 | 2310 | 73 | 38 | 95 | 133 | 71 | ? | — | Well-nourished boy, aged 22 months. Diarrhoea and vomiting began 18. vi. 14. Hyperpnoea, drowsiness. Death on 22. vi. 14. At autopsy, "status lymphaticus." |
| ,, 29 | — | — | — | — | 59 | 40 | 99 | 40 | ? | — | Very drowsy for some days, but not in coma till three hours before death |
| ,, 30 | — | — | — | — | 63 | 215 | 278 | 77 | ? | — | Coma |
| ,, 31 | — | — | — | — | 60 | 101 | 161 | 63 | ? | — | Coma |
| ,, 32 | — | — | — | — | 76 | lost | — | — | ? | — | Very drowsy but never comatose. Persistent vomiting. At autopsy, twisted ovarian dermoid |
| ,, 33 | — | — | — | — | 36 | 18 | 54 | 33 | ? | — | Blood taken just before discharge from hospital; no signs of approaching coma. Blood sugar 0·44 %; average daily sugar in urine 340 g. |
| Geelmuyden | | | | | | | | | | | |
| A.Z. | 241 | 646 | 887 | 73 | 89 | 119 | 208 | 57 | — | — | Coma. At autopsy |
| P.F. | 491 | 498 | 989 | 50 | 94 | 244 | 338 | 72 | — | — | ,, ,, |
| L.H. | — | — | — | — | 119 | — | — | — | — | — | ,, ,, |
| K.O. | 190 | — | — | — | 95 | — | — | — | — | — | ,, ,, |
| P.K. | 81 | — | — | — | 28 | — | — | — | — | — | ,, ,, |
| Marriot | | | | | | | | | | | |
| Case 6 | — | — | — | 82 | 49 | 79 | 128 | 62 | — | — | "Showed signs of impending coma" |

Marriot [1914] states that normal blood (of man, dog, pig and ox) contains less than 9·7 mg. total acetone bodies, reckoned as aceto-acetic acid, in 100 g. This result is in agreement with those given in Table II; in some of the pathological cases which give a higher figure an abnormal formation of acetone bodies may well have been in progress, since acetonuria may occur in almost any condition of disease.

*Analyses of blood from cases of diabetes.*

These are given in Table II. The cases up to No. 26 are numbered as in a previous paper [Kennaway, 1914, 2], where analyses of the urine on several days preceding death are recorded for some of them. All the cases in the table were adult diabetics, except No. 6, a diabetic boy, and No. 26, a non-diabetic infant; all the diabetics died in coma except Nos. 9, 32 and 33. All the samples of urine and blood were obtained at autopsy, except the first in case 9 and that in case 33. The estimations of alveolar $CO_2$ were made by Dr Poulton, to whom I am indebted for permission to include them here.

The term "$\beta$-ratio" was adopted in the previous paper to denote briefly the percentage of the total acetone bodies which is made up by $\beta$-hydroxybutyric acid, each substance being expressed in the form of acetone. If, in the absence of separate estimations of the acetone present, one regards this substance as aceto-acetic acid[1], the above percentage gives with sufficient accuracy the molecular proportions of the two acids, since their molecular weights differ by less than 2 %.

## Discussion of Results.

(1) Hurtley [1916] and Hurtley and Trevan [1916] have brought forward evidence to show that aceto-acetic acid has a specific toxic action, apart from its acid character, and that $\beta$-hydroxybutyric acid is a much less toxic substance both because it is a weaker acid (by eight times), and because it lacks the C.OH = CH group, which is held to be the cause of the special toxicity of aceto-acetates and of other compounds having this group (sodium salicylate, acetylacetone). One may examine the figures given in Table II to see if they have any application to this view. If one excludes the non-fatal

[1] The quantity of acetone may be by no means unimportant in this connection; in Marriot's Case 6 (see Table II) it was found to be 63 % of the acetone + aceto-acetic acid. In such estimations, however, there must always be uncertainty as to the amount of decomposition of the acid to acetone which has occurred in vitro.

case No. 33 and the non-diabetic case No. 26 the amounts of acetone bodies show the following range (see also Fig. 1):

| | Minimum | Maximum | Ratio |
|---|---|---|---|
| Aceto-acetic acid, mg. | 53 (Case 6) | 109 (Case 5) | 1 : 2 |
| $\beta$-Hydroxybutyric acid, mg. | 40 (Case 29) | 215 (Case 30) | 1 : 5·5 |

Marriot's case (in life) gave a slightly lower figure (49 mg.) for aceto-acetic acid; two out of Geelmuyden's seven results are above these limits (119 mg. aceto-acetic acid in L.H. and 244 mg. $\beta$-hydroxybutyric acid in P. F.), and one is considerably lower (28 mg. aceto-acetic acid in P.K.).

In cases 29, 30 and 31 one has three instances in which the amounts of aceto-acetic acid are almost the same (59, 63, and 60 mg.) while those of $\beta$-hydroxybutyric acid (40, 215 and 101 mg.) include the maximum and minimum given in the summary above. Fig. 1 illustrates the variations which occur in the relative amounts of the two acids.

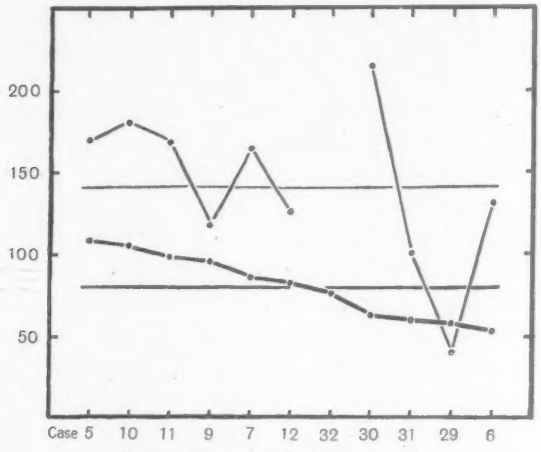


Fig. 1. Cases of Diabetes, at autopsy.
Ordinates represent acetone bodies, expressed as mg. aceto-acetic acid, in 100 cc. blood.
Upper series, $\beta$-hydroxybutyric acid.
Lower series, acetone + aceto-acetic acid; these are placed in order of magnitude
The horizontal lines represent the respective means of the two series.

If one is to derive from these results any evidence as to the relative toxicity of the two acids, it is certainly suggestive that the range of the amounts present at death is much smaller in the case of the aceto-acetic acid; differences in individual susceptibility might perhaps cause variations of 100 % in the lethal dose of a substance, but would be unlikely to explain the range of 550 % seen in the amounts of $\beta$-hydroxybutyric acid. It might be said

that the large differences in the amounts of the latter acid tolerated before the fatal issue were due to differences in the amounts of alkali administered, but the data on this point given in Table II do not show any such influence; and furthermore, this factor should lead one to look for as large variations in the amounts of aceto-acetic acid, since this is an acid eight times as strong as the other.

It is noteworthy that in the 24 hours preceding death in case 9, the $\beta$-hydroxybutyric acid underwent a distinct fall, and the aceto-acetic acid a slight increase, in amount; this might be attributed [Hurtley, 1916] to failure of the protective activity of the liver in converting the latter acid into the former. The recent experiments of Wilder [1917] show the conversion of aceto-acetic acid to $\beta$-hydroxybutyric acid (in the dog).

These results are then compatible with the view that aceto-acetic acid has a specific toxic action and are perhaps as favourable to it as can be expected from their nature; single observations cannot throw much light upon the course of a progressive intoxication. A much larger series of observations, made in each case before and during coma, and after death, together with measurements of the hydrogen ion concentration in the blood and full daily analyses of the urine, and records of the administration of alkali, are required to give any definite information on this question.

(2) Although, as was pointed out above, there is no constant relationship between the amounts of $\beta$-hydroxybutyric acid and aceto-acetic acid in the blood, the most frequently occurring proportion in this series is certainly such that there are between 1·5 and 2 molecules of the former acid to one molecule of the latter, for the $\beta$-ratio in seven of the ten fatal diabetic cases lies between 60 and 66. Case 33, in which there were no signs of approaching coma, shows a much smaller amount of acetone bodies, together with a much lower $\beta$-ratio (33), than do any of the other cases. The high proportion of aceto-acetic acid in such a case is to be expected from the results obtained with a considerable number of urines from cases of diabetes and of other conditions of acidosis [Kennaway, 1914, 2]. When the amount of the two acids is low, the amount of aceto-acetic acid is relatively high; it falls as the severity of the case increases, and tends to rise again on the approach of coma [Hurtley, 1916]. If one adopts the view that aceto-acetic acid has a special toxicity the explanation of this series of changes would seem to be as follows: if the total amount of acids is sufficiently small, the presence of a high proportion of aceto-acetic acid is of no great importance, and the $\beta$-ratio remains low; as the absolute amount rises, a protective mechanism

for the conversion of aceto-acetic into β-hydroxybutyric acid is called into increased activity, and the β-ratio rises; while the final increase in the proportion of aceto-acetic acid would indicate the failure of this mechanism (see Table II, first and second samples in case 9).

The figures for the β-ratio given in Table II show that the proportion of β-hydroxybutyric to aceto-acetic acid is always higher in the urine than in the blood[1]. One might be inclined to attribute this difference to a final accumulation of aceto-acetic acid in the blood before death, after the bulk of the urine found in the bladder at autopsy had been secreted; but the samples of blood and urine obtained from case 9 in life show the same difference. The explanation may be that the kidney receives the blood within a few seconds of its passage through the lung, where acetone has escaped; whereas the venous blood, after its slower passage through the systemic capillaries and veins, will have developed a fresh quantity of this substance.

(3) The data given in Table II enable one to form some quantitative idea of the strain which the production of these quantities of acids must place upon the maintenance of neutrality in the body.

(*a*) Suppose that a patient such as No. 5, with acetone bodies equivalent to 280 mg. aceto-acetic acid in 100 cc. blood, weighed 70 kilos and contained 3·5 litres of blood; the amount of acid in the whole blood would be 9·8 g. According to Schmidt's [1850][2] analyses, the plasma of 1000 g. of human blood contains 1·66 g. sodium. 9·8 g. aceto-acetic acid is equivalent to 2·21 g. sodium, which would be contained in the plasma of 1330 g. or 1250 cc. of blood, which is more than one-third of the whole blood in this case. Of course other bases, especially ammonia and calcium, are available as well; but some calculation of this sort may serve to illustrate the difficulties under which neutrality is maintained, or nearly maintained [Poulton, 1915] in severe diabetes.

The concentration of acetone bodies in this case (No. 5) if expressed as aceto-acetic acid, and regarded as an aqueous solution of the free acid, is nearly that of a 0·03 *N* solution.

(*b*) In case 9, the blood in life contained 243 mg. acetone bodies as aceto-acetic acid in 100 cc.; this is equivalent to 52 cc. $CO_2$ from $NaHCO_3$ at 0° and 760 mm., *i.e.* to the whole amount of $CO_2$ obtainable from this

[1] Geelmuyden's case P.F. is an exception.

[2] These seem to be the most modern full analyses of human blood, and are those which appear in most text-books. Schmidt's tables give 1·66 g. sodium in the 487 g. plasma of 1000 g. of human blood; Arronet and Warnach [1887, 1888] found 0·344 g. sodium in 100 g. of human serum. The results on plasma and serum therefore agree very closely (0·344 × 4·87 = 1·67).

volume of normal blood. The alveolar $CO_2$ tension at the time when this blood was withdrawn was found by Dr Poulton to be 17·8 mm.; if the curve given by Christiansen, Douglas, and Haldane [1914] showing the $CO_2$ content of blood in relation to $CO_2$ tension were applicable to abnormal bloods, one could obtain from these data the amount of $CO_2$ displaced from the blood by this known amount of abnormal acid, which would be of considerable interest.

(*c*) 243 mg. aceto-acetic acid (see case 9) is equivalent in molecular proportions to 214 mg. lactic acid; this amount of lactic acid, when added to 100 cc. blood, is sufficient to produce the same effect upon the dissociation constant of haemoglobin, as expressed by K, as is produced by the whole normal tension of $CO_2$ [see Barcroft, 1914, Fig. 111].

(*d*) The data given for the first eight cases in the table illustrate the part played by the kidney in the maintenance of neutrality. In this series, the mean concentration of acetone bodies in the urine (868 mg. %) is nearly four times that in the blood (230 mg. %)[1]. In case 9, the complete 24 hours' urine could not be obtained; but if its volume had been 3 litres, a common amount in such cases, and the patient's blood-volume 3·5 litres, the protective action of the kidney would have been equivalent to freeing the whole blood of acetone bodies between four and five times in the 24 hours.

In the non-diabetic case 26, the very high concentration of acetone bodies in the urine (more than seventeen times that in the blood) is noteworthy; it is no doubt due to excessive loss of water from the alimentary canal. The figures emphasise the need for a very abundant supply of fluid, given by any available method, in such cases.

(*e*) Hurtley [1916] points out that if the intoxication in severe diabetes were no more than a simple acid poisoning, such as can be produced experimentally in animals, treatment by alkalis should be more successful than it is found to be in practice. As much as 3 ounces (93 g.) of sodium bicarbonate[2]

[1] Dr Poulton has pointed out to me that the data given in the previous paper [Kennaway, 1914, 2] for case 5, in which the percentage of acetone bodies in the urine at death (483 mg.) is only 1·9 times that in the blood (279 mg.), show a fall in the concentration of acetone bodies removed by the kidney, as well as in the alveolar $CO_2$ tension, the latter change being no doubt due to the retention of increasing amounts of acid.

| July | 2 | 3 | 4 | 5 | 7 | 8 | 9 (day of death) | at autopsy |
|---|---|---|---|---|---|---|---|---|
| Total acetone bodies of urine, as gms. aceto-acetic acid per cent. | 1·53 | 1·83 | 1·42 | 1·60 | — | 0·857 | 1·04 | 0·483 |
| Alveolar $CO_2$, mms. Hg. | 27·0 | — | 26·9 | 24·5 | 17·6 | 8·8 | — | — |

[2] Such large amounts of sodium, when given without counteracting ions, may not be altogether harmless. Locke's fluid for perfusion of the mammalian heart contains Na, K, and Ca approximately in the proportions Na : K : Ca/100 : 6 : 2·5. These proportions of the three kations are

is sometimes given in a day; this is sufficient to neutralise 113 g. of the acids, which is almost equal to the amount which might be present in the whole body of a case such as No. 9, supposing the acids to be distributed uniformly throughout the blood and other body fluids (about 50 litres in a person weighing 70 kilos). As a matter of fact, this case (No. 9) did receive on each of the five days preceding death an amount of $NaHCO_3$ (70 g.) sufficient to neutralise the abnormal acids in 35 litres of blood having the composition found at this time; this would be quite 10 times the blood volume of the patient. Moreover, it is exceptional for a diabetic to produce even as much as 50 g. of the acids, as indicated by the output in the urine, in a day. There can, of course, be no doubt that the body in such cases has urgent need of the assistance of alkaline remedies, but the disappointing nature of the results certainly indicates that some additional factor, other than the simple neutralisation of acid, is involved.

The intravenous or subcutaneous injections of alkali given when coma is impending or has supervened have as a rule only a transient beneficial effect. 350 cc. of the 2 % solution of sodium bicarbonate commonly employed would suffice to neutralise an amount of the acids equal to that (8 or 9 g.) present in the whole blood-volume of a patient such as No. 9; but since, as was pointed out above, the blood may be as it were refilled with acid four or five times daily, no persistent effect is to be expected without some method of continuous injection.

## Summary.

The concentration of acetone bodies in the blood in diabetic coma may be equivalent, when reckoned as aceto-acetic acid, to that of a 0·03 *N* solution; this amount is sufficient to combine with more than one-third of the sodium of the plasma. The amounts of aceto-acetic acid show less variation in the series of cases than do those of $\beta$-hydroxybutyric acid.

Methods for the estimation of acetone and aceto-acetic acid are as a rule tested with acetone only; this is quite inadequate, as the recovery of aceto-

contained, with sufficient accuracy, in a mixture of 50 parts $NaHCO_3$, 2 parts $KHCO_3$, and 1 part $CaCl_2$; the official $KHCO_3$ is here substituted for the KCl of Locke's solution. This simple ratio could be applied in the prescription of any desired amount of $NaHCO_3$ for administration by the mouth, *e.g.*:

| | |
|---|---|
| Sodium Bicarbonate | 150 grains |
| Potassium Bicarbonate | 6 „ |
| Calcium Chloride | 3 „ |

This solution is of course turbid from the precipitation of $CaCO_3$, but the $CaCl_2$ might be given at a different time from the rest.

acetic acid is liable to an error which does not occur in the case of either acetone or $\beta$-hydroxybutyric acid.

The expenses of this investigation were defrayed by a grant from the Science Committee of the British Medical Association.

I am indebted to Dr Turnbull, of the London Hospital, for the material from three of the cases of diabetic coma.

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# XI. THE ANTI-SCORBUTIC VALUE OF COW'S MILK[1].

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## Introduction.

The experiments described in the following paper form a small group out of a much larger scheme. The main scheme was planned in order to obtain a comparison between the anti-scorbutic values of a number of foodstuffs, in response to war-time requirements, both civil and military, within the British Empire. Some of the preliminary results have already been published [Chick and Hume 1917; Chick, Hume and Skelton 1918] owing to urgent practical need for their application on active service and at home, and it was intended that a detailed report of the whole work should appear later, when the investigation was complete.

We believe, however, that any contribution to the study of the nutritional value of milk may be of immediate practical value in connection with the problems of infant feeding, particularly at the present time. The following experiments are by no means complete or exhaustive, but, though few in number, they form a complete and concordant series. Work of this character involves great labour and the experiments with milk have had temporarily to be abandoned to permit time and attention to be devoted to other, equally important foodstuffs. It has therefore seemed wisest not to delay publication of the results already obtained.

It has been usual to regard fresh cow's milk as a substance possessing valuable anti-scorbutic properties and capable of preventing scurvy, if present in a diet in even moderate amount. This view is held by many authorities on infant feeding. There does not, however, appear to be any sound basis for such an opinion and though direct evidence upon this point is

[1] A short summary of this paper appeared in the *Lancet*, Jan. 5th, 1918

scanty, what there is, is opposed to such a view. Curran [1847] in his description of the scurvy epidemic in Dublin in 1847, cites more than 80 cases among Union inmates, all of whom had received one pint of milk daily for at least six months before falling ill. The diet in the Unions, however, was deficient in fresh meat and vegetables, and potatoes had been scarce, owing to the potato famine.

More recent evidence upon this point is derived from careful experience of infant feeding. The observations of Hess and Fish [1914], Hess [1916], Miller [1917] and others also suggest that cow's milk is a food-stuff comparatively poor in anti-scurvy substances. They indicate further that many methods of preparation of food for the use of infants, such as heating, drying, etc., tend to diminish the small amount of these valuable constituents originally present, with a distinct resultant risk of causing slight and incipient infantile scurvy. The results of our experimental work are in accord with the observations of these workers, which deserve wide publication and will be referred to in detail later, see page 150.

*Experimental.*

From the close analogy in etiology, symptoms, method of cure and post-mortem appearances in case of death, guinea-pig scurvy has generally been accepted as the equivalent of human scurvy by those having experience of both conditions. The guinea-pig has been selected for the study of experimental scurvy on grounds of general convenience and because this animal is particularly susceptible to the disease. If all fresh vegetable food is removed from a young, growing guinea-pig on a diet of grain and water, scurvy symptoms will appear in about 20 days and death will occur in about a month (see Table I and Chart 1). The anti-scorbutic value of various foodstuffs can be estimated by adding these in varying amounts to the scurvy diet of grain and water.

Guinea-pigs were used by Holst and Frölich [1912] in their classic work on experimental scurvy and by Frölich [1912] who specially investigated cow's milk. He found that these animals could be protected from scurvy by an exclusive diet of fresh milk and that raw milk was a better diet than heated milk. It is difficult to interpret his experiments fully, as no indication is given of the quantities consumed.

Funk [1913] states that 50 cc. of fresh unboiled milk daily, in addition to oats, prevented loss of weight and onset of scurvy in guinea-pigs, a result

which we have not succeeded in confirming. His experiments are not, as far as we are aware, set out anywhere in detail.

Jackson and Moore [1916] found that of guinea-pigs fed on pasteurised milk *ad libitum*, 5 remained well out of 32; on raw milk, out of 9, all died. McCollum and Pitz [1917] fed guinea-pigs on oats and fresh cow's milk *ad libitum*, and found that some survived and some died. In both these researches no figures are given for the amounts of milk actually consumed by the animals. The present work is therefore the first in which a special effort has been made to include the quantitative factor.

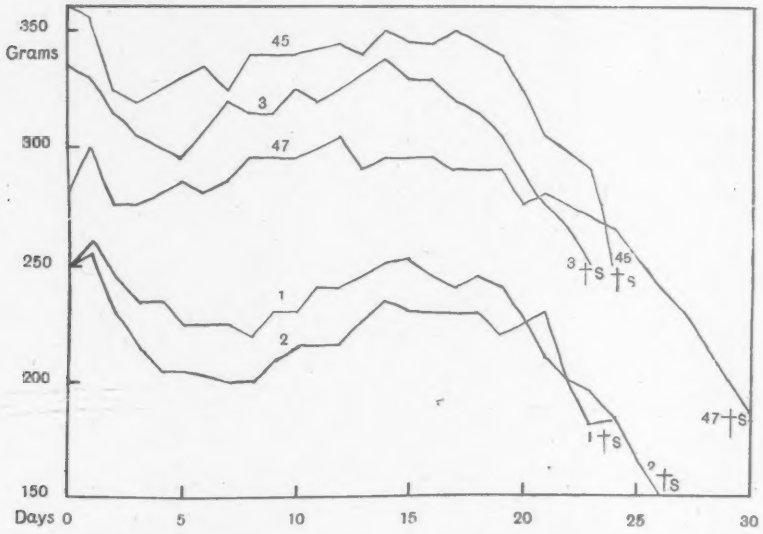


Chart 1. Weight curve of young guinea-pigs upon a diet of oats and bran (*ad lib.*) and water, see Table I. †s signifies death from scurvy.

The general course of events in an unprotected guinea-pig, when grain and water alone are given, is seen on reference to Chart 1 and Table I. The details of 5 young guinea-pigs fed upon oats, bran and water *ad libitum* show a considerable degree of consistency. The initial weight, 250–360 grams, is maintained, but barely exceeded, for a period of 15 to 18 days (see Chart 1), after which it gradually falls till at death the loss is 25–30 % of the original. Definite scurvy symptoms were noted after periods of 17–22 days; and death from scurvy occurred between the 23rd and 30th day.

TABLE I. *Development of Scurvy in Young Guinea-pigs upon a diet of Grain and Water.*

| No. of experiment | No. of animal | Diet | Weight of animal, g. At beginning | Highest reached | At end | Change in weight, % | Length of experiment, days | Day on which symptoms of scurvy were first noted | Result | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | Oats, wheaten bran, water, *ad lib.** | 250 | 260 | 182 | −37 | 24 | uncertain | Death from scurvy, | 24th day |
| 2 | 2 | do. | 250 | 250 | 150 | −40 | 26 | 22 | do. | 26th ,, |
| 3 | 3 | ,, | 335 | 338 | 250 | −25 | 23 | 17 | ,, | 23rd ,, |
| 4 | 45 | ,, | 360 | 360 | 250 | −31 | 24 | 21 | ,, | 24th ,, |
| 5 | 47 | ,, | 280 | 300 | 185 | −34 | 30 | 17 | ,, | 30th ,, |

* In health the animals consumed about 30–50 g. oats and bran daily; when sickening with scurvy the amount decreased to 10–20 g. or less.

The use of a basal diet of oats, bran and water is open to the criticism that it is deficient in another accessory food-factor besides the anti-scorbutic one, *i.e.* the "Fat Soluble A" of McCollum and others, and that therefore any experiments performed with it will be subject to two limiting factors instead of one. We have therefore made it our general custom in studying experimental scurvy to add to the diet of oats and bran, 60 cc. a day of milk autoclaved at 120° C. for an hour: of this from 30 to 60 cc. were usually consumed. We have not found that this addition delays the onset of scurvy symptoms but it does alter the character of the weight curve. If Chart 1 (oats, bran and water) be compared with Chart 2 (oats, bran and autoclaved milk), it is seen that while on the first diet, weight is maintained till the first onset of scurvy symptoms, on the second diet there is a steady gain in weight for about the same period.

This effect of milk in maintaining growth after it has been deprived of its anti-scorbutic vitamine is interesting in view of the results obtained by Hopkins [1912] and later by McCollum and Davis [1913] and by Osborne and Mendel [1913] in their experimental work on growth. The accessory growth factor, termed "Fat Soluble A," of which one important source is butter fat, would appear to be operating in our autoclaved milk, and to be distinct from the anti-scorbutic factor.

Further experiments are in progress in order to test whether this explanation is correct or whether the influence of the milk ration in promoting growth is, in this case, due simply to its content of fat, protein and salts.

These American investigators, working with young rats, have arrived at the conclusion that, in addition to adequate provision of protein, carbohydrate, fat and salts, for nutritive purposes two accessory factors are required, if satisfactory growth is to be maintained. The first is termed "Fat Soluble A" [McCollum and Kennedy 1916], and is to be found among other sources in butter fat and egg fat [McCollum and Davis 1913], in beef fat [Osborne and Mendel, 1915], in codliver-oil [Osborne and Mendel, 1914], and in some green leaves [McCollum, Simmonds and Pitz, 1918]. The second "Water Soluble B" [McCollum and Kennedy, 1916], of which rich deposits are found in the germ of cereals [McCollum and Davis, 1915] and in yeast [Funk and Macallum, 1915], is considered [McCollum and Kennedy, 1916] to be identical with the accessory

factor (anti-beri-beri or anti-neuritic vitamine) protecting from avian polyneuritis and human beri-beri. McCollum and his co-workers do not admit that there is an additional factor protecting from scurvy. It is however possible that had their work been carried out with another experimental animal they might have reached another conclusion. It is well known that the rat is insusceptible to scurvy, and it may well be that it requires only a small amount of the anti-scorbutic factor and that sufficient traces may remain in the substances of animal and plant origin which supply the two "growth" factors.

Our work leaves us in no doubt that, in the case of guinea-pigs and man, an accessory anti-scorbutic factor is required to maintain health. Although in many cases growth will cease in young animals if this anti-scorbutic factor is omitted from the diet, it does not appear likely from a study of their respective distributions that this anti-scurvy vitamine is identical with either of the two growth factors described by the American workers. In our own experiments, the anti-beri-beri vitamine (probably identical with the growth factor "Water Soluble B") is present in abundance in the ample ration provided of whole oats and wheaten bran. The second growth factor, "Fat Soluble A," may be operating in our milk ration (even when strongly heated) and is distinct from the anti-scurvy accessory factor.

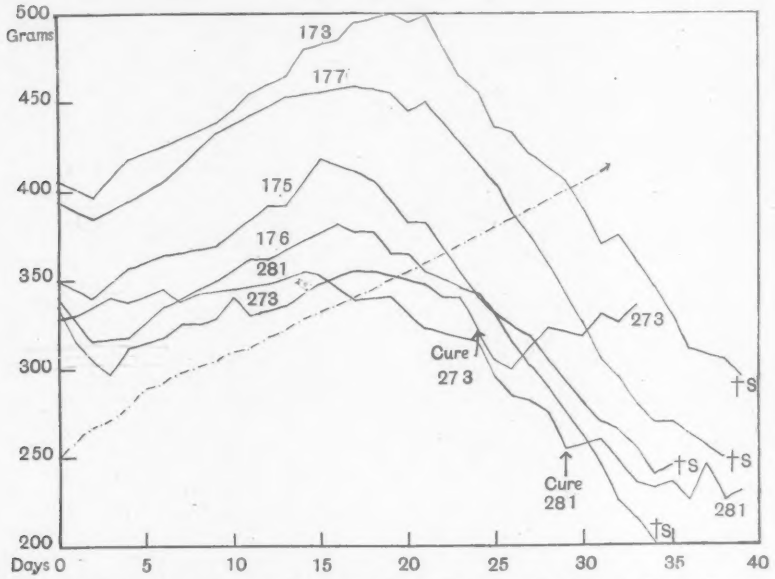


Chart 2. Weight curve of young guinea-pigs upon a diet of oats and bran (*ad lib.*) and autoclaved (1 hour at 120° C.) cow's milk up to 60 cc. daily, see Table II, Expts 1–6.

·—·—·— signifies normal weight curve upon a diet of oats and bran and fresh cabbage *ad lib.*

†s signifies death from scurvy.

↑ Cure signifies cure begun with fruit juice.

It is necessary to give a short description of what is meant in the Tables by "scurvy symptoms." The first symptom to be observed is soreness of joints and limbs, more especially of shoulders and knees, so that the animal squeaks when pressure is applied to these places. Some animals squeak when

TABLE II. *Influence of addition of* (*a*) *strongly heated* (120° C. *for one hour*) *milk and* (*b*) *dried milk, upon Scurvy in Guinea-pigs upon diet of Oats and Bran compared with* (*c*) *other anti-scorbutic materials.*

| No. of experiment | No. of animal | Diet: Average daily ration of oats and bran | Diet: Average daily ration of milk, cc. (*a*) *Autoclaved milk* 1st 21 days | Diet: Average daily ration of milk, cc. (*a*) *Autoclaved milk* Later | Weight of animal, g. At beginning | Weight of animal, g. Highest reached | Weight of animal, g. At end | Weight of animal, g. Change in weight, % | Length of experiment, days | Day on which symptoms of scurvy were first noted | Result |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 173 | oats and bran, *ad lib.* | 60 | 55 | 405 | 500 | 298 | −26 | 40 | 21 | Death from scurvy, 40th day |
| 2 | 175 | ,, ,, | 45 | 20 | 347 | 418 | 200 | −43 | 34 | 21 | ,, ,, 34th ,, |
| 3 | 176 | ,, ,, | 41 | 40 | 340 | 382 | 245 | −28 | 35 | 21 | ,, ,, 35th ,, |
| 4 | 177 | ,, ,, | 47 | 32 | 395 | 458 | 240 | −39 | 37 | 18 | ,, ,, 37th ,, |
| 5 | 273 | ,, ,, | 45 | 34 | 339 | 355 | 320 | − 6 | 24 | 15 | In condition of acute scurvy, 24th day, cure with lemon juice started |
| 6 | 281 | ,, ,, | 42 | 22 | 330 | 356 | 255 | −23 | 29 | not noticed | "Cure" with fresh lime and fresh orange juice started 29th day |
| | | | (*b*) *Dried milk* | | | | | | | | |
| 7 | 163 | ,, ,, | 60* | | 346 | 373 | 237 | −32 | 34 | 28 | Death from scurvy, 34th day |
| 8 | 162 | ,, ,, | 76* | | 350 | 395 | 350 | 0 | 23 | 21 | "Cure" with preserved lemon juice started 23rd day |
| | | | (*c*) *Other anti-scorbutic materials* | | | | | | | | |
| 9 | 440 | *ad lib.* + 2·5 g. fresh cabbage | ca. 60 | | 320 | increasing to | 572 | +79 | 95 | — | Good health |
| 10 | 423 | *ad lib.* + 5 g. fresh cabbage | ca. 60 | | 299 | ,, | 565 | +89 | 92 | — | ,, ,, |
| 11 | 239 | *ad lib.* + 3 cc. fresh orange juice | ca. 60 | | 365 | ,, | 598 | +64 | 93 | — | ,, ,, |
| 12 | 103 | *ad lib.* + 10 cc. fresh raw beef juice | 0 | | 350 | 350 | 240 | −31 | 23 | 15 | Death from scurvy, 23rd day |

* Reckoned as the equivalent of ordinary milk.

handled under any circumstances, and when in perfect health; but if they are examined regularly from the beginning of the experiment, it is possible to distinguish those which are feeling pain. The presence of painful members is also shown by the assumption of what we have called the "scurvy position," which seems to indicate haemorrhage and consequent discomfort in the muscles of the limbs. The animal rests on its side, and the painful leg is held off the ground and may be seen twitching. A second attitude, which we have called the "face-ache position," is also indicative of scurvy in young guinea-pigs; the animal lies curled up with the side of its face pressed on the floor of the cage. This is a frequent attitude in adult guinea-pigs when in normal health, but we have never seen a young animal adopt it except when ill with scurvy. It seems to indicate haemorrhage of the jaw, with soreness and looseness of the teeth. The state of the molar teeth and of the whole gums cannot be inspected during life, and it is only possible to judge of the condition by the greater or less capacity for eating, and by the assumption of the "face-ache position."

*Post-mortem appearances.* The macroscopic signs of scurvy in guinea-pigs consist mainly of haemorrhage and bone lesions and have been well described by Holst and Frölich [1907, 1912]. It may be convenient to recapitulate them here.

The haemorrhage may occur in almost any situation; for example, in the subcutaneous connective tissue, especially in the region of the axilla or groin, and over the ribs, or among the muscles, more frequently of the upper and lower limbs, and less often in the intercostal muscles. In chronic cases of long standing, the whole musculature appears darker in colour than in the normal animal. Haemorrhages in the walls of the viscera are also frequent; in these cases blood is often passed with the faeces during life and death occurs suddenly.

The changes in the bony tissues are characteristic, and are conveniently observed in the knee joints. In a severe case these are very much swollen, and on dissection the tibial epiphysis (or more rarely the femoral) is found to be disorganised and often broken across; the condyles can best be described as papery, the shafts of the long bones are brittle and can be easily broken with forceps, and the marrow is much darker than the normal. Periosteal haemorrhages, which form so marked a feature of infantile scurvy, are seen in comparatively few cases. The jaws and teeth are usually profoundly affected, the teeth become brittle and loose, and the jaw is easily fractured on pressure.

Typical changes take place in the costo-chondral junctions. They become swollen, and exhibit a transverse yellow bar and are frequently surrounded with haemorrhages in the adjoining muscles. These appearances correspond with the complete disorganisation of the bone-cartilage junction, and with disappearance of the cartilaginous trabeculae and of the rows of cartilaginous growing cells. In many cases the bone is found to be completely fractured and there is great proliferation of connective tissue across the junction.

*Experiments with fresh Cow's Milk.*

GROUP I.

The experiments with fresh milk fall into two groups.

The first group of experiments consists of those in which not more than 30 to 50 cc. of fresh milk were presented to each animal daily, together with an *ad libitum* (measured) ration of oats and bran. The larger quantity of milk was given under the impression that we should protect our animals from scurvy by such a ration [Funk, 1913]. This, however, proved not to be the case, and in every instance acute scurvy was the result.

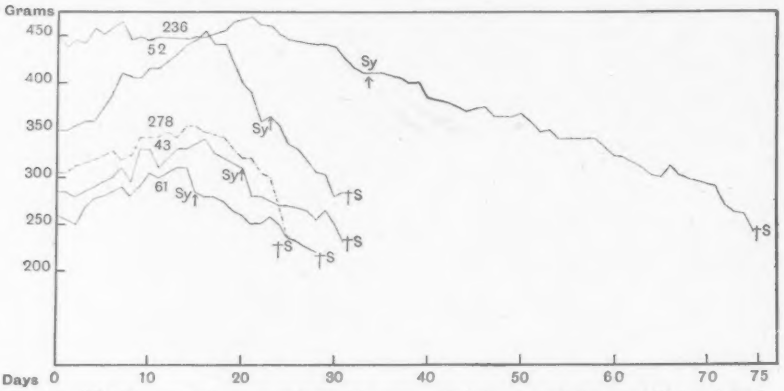


Chart 3. Weight curve of young guinea-pigs upon a diet of oats and bran (*ad lib.*) and fresh cow's milk 30–50 cc. daily, see Table III. In case of No. 236, the daily ration of 50 cc. was maintained continuously by hand-feeding.

Sy ↑ signifies scurvy symptoms noted. †s signifies death from scurvy.

·—·—·— signifies 1 cc. liquid paraffin given every second day.

The details concerning the 8 animals in this group are set out in Table III and Chart 3, and present a very uniform result. All (with the exception of No. 236, described later) developed scurvy symptoms in 15 to 28 days, an average time not very much longer than when the milk was omitted (see Table I). In cases where no treatment was essayed, the symptoms became progressively severer, great loss of weight occurred, and the animals became moribund about 10 days later. They were usually killed by chloroform at this stage (Nos. 1, 2, 4, 7, Table III).

In the case of other animals in this group fresh fruit juices were given after definite scurvy symptoms had been observed, with the result that successful cures were obtained. The animals, after a short interval, began to

TABLE III. *Influence of Daily Ration of* 30–50 *cc. fresh Cow's Milk upon Scurvy in Guinea-pigs. The diet consisted otherwise of Oats and Bran* ad libitum, *the amount of which consumed varied between* 20 *and* 40 *g. daily.*

| No. of experiment | No. of animal | Daily ration of milk offered, cc. | Weight of animal, g. At beginning | Highest reached | At end | Change in weight, % | Length of experiment, days | Day on which symptoms of scurvy were first noted | Result | Post-mortem examination result |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 43 | 30 | 285 | 340 | 215 | −25 | 31 | 20 | Very ill, killed 31st day | Scurvy |
| 2 | 44 | 30 | 290 | 295 | 205 | −29 | 29 | not noticed | Moribund, killed 29th day | „ |
| 3 | 51 | 50 | 320 | 375 | 260 | −19 | 31 | 20 slight 27 severe | "Cure" 5 cc. fresh orange juice daily started 31st day. Killed 77th day in fair health. Weight = 420 g. | Traces of old scurvy |
| 4 | 52 | 50 | 350 | 450 | 285 | −18 | 31 | 28 | Very ill, intestinal haemorrhage. Killed 31st day | Scurvy |
| 5 | 53 | 50 | 355 | 410 | 290 | −18 | 31 | 20 | "Cure" 5–10 cc. fresh orange juice daily started 31st day. Splendid condition 90th day. Weight = 470 g. Used for breeding | |
| 6 | 60 | 50 | 280 | 385 | 340 | +21 | 17 | 18 | "Cure" 10 cc. fresh orange juice daily, 18th day. Killed 63rd day, in fair health. Weight = 450 g. | Traces of old scurvy |
| 7 | 61 | 50 | 260 | 310 | 220 | −15 | 30 | 15 | Death from scurvy, 30th day | Scurvy |
| 8 | 278 | 60 + 1 cc. liquid paraffin each second day | 305 | 355 | 237 | −22 | 25 | not noticed | „ „ 25th „ | „ |
| 9 | 277 | „ | 318 | 335 | 295 | −7 | 24 | 15 | "Cure" attempted with lime juice beginning 24th day | Traces of old scurvy |

put on weight, the teeth, if loose, began to tighten up, and very slowly the soreness of the joints began to improve. The animals were killed after varying periods of time, and, although in good health, traces of the old scurvy were demonstrable even when some months had elapsed since the date of acute symptoms. These traces consisted in survival of the bone changes in acute scurvy, such as swellings at costo-chondral junctions, and malformation of leg bones with bony outgrowths due to partial or complete separation of the tibia at the upper epiphysis and subsequent joining up again of the fractured bone in the wrong position. Disturbance in the position of the teeth was also sometimes noted.

No animal in this group failed to develop scurvy. The ration of milk offered (*Expts.* 1, 2, 30 cc.; *Expts.* 3 to 9, 50 to 60 cc.), was usually consumed as long as the animal remained well; with the onset of scurvy symptoms the amount of milk taken decreased until the end of the experiment, 10 to 15 days later. The supply of anti-scurvy accessory substance is here so deficient that individual idiosyncracy and power of resistance play an insignificant part. In our experience no guinea-pig can remain in health over a long period upon a diet of oats and bran, to which as little as 30 to 50 cc. of fresh milk daily is added.

### Group II.

In the second group of experiments, the amounts of fresh milk consumed were greater, viz.—from 50 cc. upwards.

This group (see Table IV, Charts 3 (No. 236) and 4) contains few animals, but the nature of the work made this unavoidable. Throughout the scurvy investigation in the Institute the rule has been that the animals are not left to the care of the laboratory servants, but are handled and weighed by the worker in charge, who also measures the amount actually consumed of each constituent of the diet. We have found that this is the only way in which a really satisfactory knowledge of the working of the experiment can be gained, although the amount of the daily routine is greatly multiplied. In addition to these precautions, it was often necessary, with the animals in the second group, to reinforce with hand-feeding, in order that the standard of milk consumption should be kept up. It is not, however, possible to give more than about 50 cc. of milk by hand during the day and, unless the animal also cooperates heartily in the experiment during the night, the total daily consumption does not rise above 70–80 cc.

The ration of oats and bran was cut down to a minimum, 10 g. in order

TABLE IV. *Influence of Daily Ration of 50–130 cc. fresh Cow's Milk upon Guinea-pig Scurvy.*

| No. of experiment | No. of animal | Average daily ration of milk consumed, cc. | *Ration of oats and bran, g. | Weight of animal, g. At beginning | Weight of animal, g. Highest reached | Weight of animal, g. At end | Weight of animal, g. Change in weight, % | Length of experiment, days | Day on which symptoms of scurvy were first noted | Result | Post-mortem examination results |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 236 | 50† | *ad lib.* | 451 | 469 | 240 | −47 | 75 | 33 | Very ill, scurvy. Killed 75th day | Scurvy |
| 2 | 232 | 65 | *ad lib.* | 467 | 507 | 382 | −18 | 39 | 28 | Accidentally killed 39th day | ,, |
| 3 | 234 | 1st period, 14 days 68<br>2nd ,, 32 ,, 57<br>3rd ,, 34 ,, 73 | 10 | 275 | 340 | 268 | −2 | 83 | 21 | Moribund, killed 83rd day | ,, |
| 4 | 242 | 1st period, 44 days 86<br>2nd ,, 21 ,, 57<br>3rd ,, 24 ,, 61 | 10 | 453 | 490 | 300 | −34 | 91 | 45 | Death from scurvy, 91st day | ,, |
| 5 | 243 | 85 | *ad lib.* | 480 | gradually increasing to | 555 | +16 | 24+ | never | Good health, birth of 2 young on 24th day | No scurvy |
| 6 | 235 | 1st period, 22 days 84<br>2nd ,, 31 ,, 132<br>3rd ,, 36 ,, 129<br>4th ,, 30 ,, 124 | 10 | 260 | ,, | 586 | +125 | 113+ | never | Good health | — |

* In health the animals fed *ad lib.* consumed 30–50 g. daily; when sickening with scurvy the amount decreased to 10–20 g. or less.

† This animal did not voluntarily take as much as 50 cc. daily, the amount not consumed was fed by hand, so that the daily consumption was regularly 50 cc.

that a sufficient volume of milk could be accommodated; we also found that a moderately high temperature of the laboratory was helpful in increasing thirst. In all the experiments the milk was given twice daily. At first it was ordinary dairy milk, but much trouble was encountered through souring during the night, after which most animals refused to touch it. For a short time boracic acid (0·5 g. per litre) was added to the night milk to preserve it, but this did not improve the animals' appetite. For the latter part of the time, in the experiments of this group, very pure, specially delivered, country milk was used. No further trouble was experienced with souring.

The usual procedure was to offer 50 cc. of milk in the morning which had to be consumed during the day, with the help of some hand-feeding, if necessary. At night, a measured amount, graded to the particular appetite of the animal in question, was offered, and the residue was carefully measured next morning.

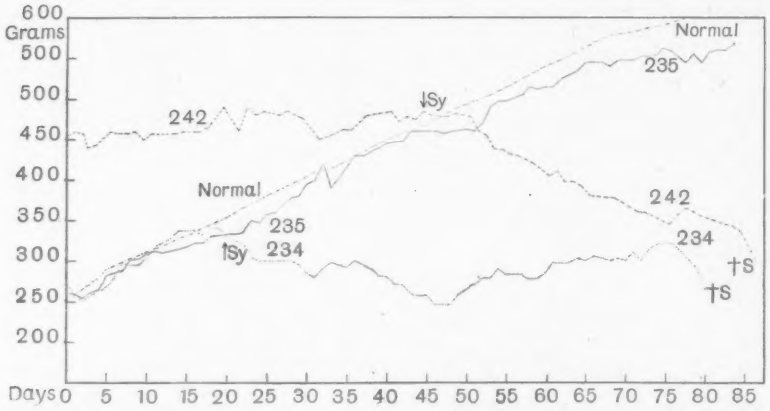


Chart 4. Weight curves of young guinea-pigs upon a diet of oats, bran and fresh cow's milk 50–130 cc. daily, see Table IV: No. 234, 60–80 cc., No. 242, ca 80 cc., No. 235, 80–130 cc.

· — · — · — signifies normal weight curve upon a diet of oats, bran and fresh cabbage *ad lib.*

†S signifies death from scurvy.

↓Sy signifies scurvy symptoms observed.

The details concerning animals in Group II are set forth in Table IV, and the weight charts of three typical cases are shown in Chart 4; the animals themselves, however, deserve individual notice.

No. 236 (initial weight 450 g.), offers an interesting contrast with the animals of Group I, and its weight chart is set out in Chart 3, for purposes of comparison with the animals of that group. This animal consumed exactly 50 cc.

of milk daily throughout the experiment. Incipient symptoms of scurvy appeared about the 33rd day, and a gradual decline of weight set in, but the animal did not become acutely ill until the 75th day. This is in marked contrast with the other animals in Group I, and is attributable to the fact that the daily ration of 50 cc. fresh milk did not fall off when the animal became ill, but was fed daily to it by hand.

The weight curve of No. 236 we regard as characteristic of partial protection against scurvy. The anti-scorbutic accessory factor is present in the diet but in insufficient quantity and the life of the animal is prolonged, though it ultimately succumbs with symptoms of scurvy of long standing.

No. 232 (initial weight 467 g.), consumed an average of 65 cc. milk, and did well for a time. About the 22nd day it showed symptoms of scurvy, and its weight fell rather fast. At this period even with hand feeding it was occasionally receiving less than 50 cc. of milk daily. About the 30th day it became very thirsty and consumed 70 to 80 cc. daily and the weight improved. Unfortunately the animal became enfeebled with an attack of diarrhoea, its daily consumption of milk reached 100 to 110 cc., but in its eagerness it unfortunately fell into its milk pot and was drowned.

No. 234 (initial weight 275 g.). The average daily consumption of milk varied from 68 cc. for the first two weeks, to 57 cc. for the next four weeks, and 73 cc. for the last five weeks. The weight of the animal was well maintained throughout the 83 days of the experiment, but symptoms of scurvy were apparent from the 21st day.

No. 242 (initial weight 453 g.). The average daily ration of milk was at first greater than in case of 234, viz., about 86 cc.; later it fell below it. On the whole, however, the result corresponded very nearly with that of No. 234 though the animal was much larger to begin with. Scurvy symptoms were developed at the end of the third week and death supervened after 91 days.

No. 243 (initial weight 480 g.) did well, consuming an average of 85 cc. and increased in weight until the 25th day, when two living young were born. It immediately fell ill and died of a general septicaemia a few days later. No symptoms of scurvy were observed in life or at the post-mortem examination.

No. 235 (initial weight 260 g.) was started at the same time as, and in a manner in every way similar to, No. 234. From the first it co-operated well in the experiment and took its milk with enjoyment. It needed very little hand feeding. For three weeks it consumed an average of 84 cc. and put on weight steadily; later its daily average increased to about 120 cc., while on some days it consumed as much as 200 cc. It survived for more than 16

weeks; it more than doubled its weight, and during this period showed no symptom of scurvy.

The results of experiments in Groups I and II taken together suggest that it is the *quantity* of fresh milk consumed which is the important factor in influencing the survival of guinea-pigs upon a diet of oats, bran and fresh milk. If these results be compared with those summarised in Table I, where milk was omitted and where the guinea-pigs subsisted on oats, bran and water only, or with Table II where oats and bran and autoclaved milk were given, the following conclusions are reached with regard to the anti-scorbutic value of fresh milk:

1. When less than 50 cc. are taken daily, the animals showed no protection from scurvy.

2. When exactly 50 cc. are taken (No. 236) some degree of protection was apparent, life was prolonged, but the weight declined from a comparatively early date.

3. When 50–85 cc. are taken, increased protection from scurvy was noticed, and weight was better maintained (Nos. 232, 234, 242, 243).

4. In the one instance where 100–150 cc. was the daily ration (No. 235) the animal grew in a manner closely approximating to the normal (see Chart 4) and showed no symptoms of scurvy for 16 weeks.

Our interpretation of these results leads us to a concept of fresh cow's milk as a substance containing the anti-scorbutic accessory substance, but in small amount. It is therefore necessary that a large daily ration should be consumed, in fact that the diet should be composed entirely or almost entirely of milk, if scurvy is to be prevented by its agency alone. Such a concept is consistent with the rôle played in nature by mammalian milk, which is destined to form a complete food for a definite, but limited, period for the young of the species.

The low anti-scorbutic value of fresh milk is realised from Table II (*c*), where results obtained with more potent substances are given for purposes of comparison. It will be seen that scurvy in guinea-pigs can be effectively prevented by a daily ration of 2·5 to 5 g. fresh cabbage leaves, or 3 cc. fresh orange juice, whereas upwards of 100 cc. fresh cow's milk is required.

Our experimental work with fresh milk thus falls into line with other work pointing to scurvy as a "deficiency disease," caused by absence from, or deficiency in, the diet of an accessory food factor.

A similar set of experiments (already alluded to, p. 133), have recently

been published by McCollum and Pitz [1917] and results resembling our own were obtained. The interpretation placed upon them by these authors was, however, entirely different from our own and led them to reject altogether the "vitamine hypothesis" of guinea-pig scurvy. They arrive at the conclusion that this disease is not due to any deficiency in the diet, but that it is the result of chronic constipation, caused by the physical structure of a "scurvy diet."

They are led to this conclusion by the fact that a certain diet, *i.e.*, rolled oats, casein, salts and butter fat (the last-named supplying the accessory growth factor, "Fat Soluble A") sufficient to maintain the rat in perfect health, is insufficient to maintain the guinea-pig. The latter goes down hill and dies, but whether from starvation and general inanition, or whether from the symptoms described by Holst and Frölich [1907, 1912] and other workers as scorbutic, *i.e.* haemorrhages, fragility of the bones and loosening of the teeth, together with specific histological changes in bone and bone marrow, is not stated. A further set of experiments with guinea-pigs is described, in which the diet consisted of rolled oats and fresh milk. In this case a certain proportion (two out of four) died of scurvy in 5–7 weeks; the remaining two animals survived, and it is argued that if some survive the diet must be chemically adequate for all. No allowance is made for individual idiosyncracy or for possible differences in the amounts consumed.

McCollum and Pitz consider that the metabolism of any two mammals, *e.g.*, the rat and the guinea-pig, must be similar, so that a diet which is chemically sufficient for the one must be chemically sufficient for the other, and if the one animal fails on it while the other survives, the failure must be due to some other cause than the deficiency in diet. They repudiate the idea that the guinea-pigs die from a "deficiency" scurvy and they attribute the deaths which occur on these diets to chronic constipation. They consider that the guinea-pig is peculiar in needing foods which will produce bulky easily eliminable faeces, and oats and milk they do not regard as belonging to such a category. They find support for this view in the fact that post-mortem examinations of guinea-pigs dead of scurvy generally show an empty stomach and a gorged caecum; further, they consider that scurvy in guinea-pigs can be both prevented and cured by the administration of laxatives, without any other alteration of the diet.

The results of experimental work carried out in this Institute [Chick and Hume, 1917] in confirmation of that of Holst and Frölich [1907, 1912, 1913], Fürst [1912], etc., in Christiania, have furnished abundant data in support

of the explanation of guinea-pig scurvy as a deficiency disease, analogous as regards etiology, symptoms and methods of cure with the condition called scurvy in man. And, furthermore, the results which we have obtained by feeding guinea-pigs on common articles of human diet, including milk, correspond in a striking manner with current experience and old records of human scurvy.

We believe that the experiments of McCollum and Pitz will bear an entirely different interpretation from that which they have put upon them, an interpretation which falls into line with the "vitamine" hypothesis and requires no assumption of constipation as the causative agent. This interpretation we believe to be as follows: those guinea-pigs (2 out of 4) in their experiment which survived on a diet of unlimited fresh cow's milk and oats were those which consumed a large quantity of milk and very little oats, while those which developed scurvy were those that consumed much less milk. It is clear that an *ad libitum* ration of fresh milk may mean anything and as the quantities consumed were not measured, the above explanation could not emerge.

There remains the case of those animals developing scurvy on a diet of fresh milk and oats, whose condition improved after regular administration of a laxative [*e.g.* McCollum and Pitz, 1917, Nos. 2 and 4, Chart 7, p. 249]. A fall in weight coinciding with scurvy symptoms at about the 5th week, was checked and the animals did well subsequently. It will be found that weight charts, closely resembling these, are to be found where no curative doses were given [*e.g.*, McCollum and Pitz, 1917, Chart 3, p. 245, Chart 4, p. 246]. We have noticed that a distaste for solid food and greatly increased appetite for milk frequently accompanies an attack of scurvy. The improvement in weight following a fall, which was noticed in cases where a laxative was administered (and recorded also, without comment, in some cases where none was given) may possibly have corresponded to an increased consumption of the fresh milk. In our own experiments the length of life and welfare of the animals were found to be nicely graded to the amount of milk taken, and had McCollum and Pitz recorded the amount of milk actually consumed by their animals, we believe that their work would have yielded the same result.

As regards the constipation hypothesis of McCollum and Pitz, we have not observed that this condition is a constant feature of guinea-pig scurvy, indeed we should have said that diarrhoea with intestinal, duodenal and sometimes gastric haemorrhage, entered more often into the clinical picture. In some

cases it is true, the failure to eat resulting from loosened teeth, does lead to a post-mortem picture showing an empty stomach and small intestine, and a stagnant caecum. No doubt cases of constipation occur in this way, but we have not found them the regular concomitant of scurvy symptoms. On the other hand many facts have emerged from the work in this Institute which are quite incompatible with the view that scurvy in guinea-pigs is caused by a chronic state of constipation.

Dr E. Marion Delf[1] has found that guinea-pigs can be protected from scurvy by 5 g. of peas, germinated for 48 hours [cf. Fürst 1912] when added to a diet of grain and milk (autoclaved at 120° for an hour). A very much larger ration, about 30 g., is needed to do this when the peas are merely soaked in water for 24 hours. It is hard to believe that the process of germination, which has proceeded for so short a time, can so alter the physical properties of a diet that its power of preventing or curing constipation should have increased sixfold.

McCollum and Pitz refer to Holst and Frölich's [1912] experiment with dried greens and suggest that the loss of power to prevent scurvy, which cabbage suffers on drying, is due to the failure of the greens to take up water in the intestine and to produce the bulky food needed to prevent constipation. We have repeated Holst and Frölich's work with cabbage, dried and kept for three months. Before being fed to guinea-pigs, the greens were soaked and became fully re-imbibed, yet an amount equal to more than 20 times the minimal ration of fresh cabbage was found incapable of preventing scurvy.

The changes in the food stuffs which are described in these two cases are not mere changes in physical condition but appear much more likely to be subtle chemical changes involving the appearance or disappearance of an, at present unisolated, chemical complex, such as a "vitamine" or accessory food factor is conceived to be.

It appeared worth while, however, to put the matter to an experimental test. Guinea-pig No. 278 (Chart 3 and Table III) received oats, bran and 50 cc. fresh milk daily; in addition 1 cc. liquid paraffin was administered every second day. This animal behaved like those which received a similar ration of fresh milk without any laxative. A fall in weight began on the 15th day, scurvy symptoms occurred on the 27th day.

In a second experiment guinea-pigs 269 and 270 were fed on a scurvy diet of oats, bran and autoclaved milk; 1 cc. liquid paraffin was given every second day, beginning from the 23rd day, the date at or about which scurvy

[1] Unpublished experiments.

symptoms were noticed. A few days later a steady fall in weight set in and death from scurvy took place on the 39th and 50th days respectively. The weight charts of these animals are set out in Chart 5, and their histories should be compared with those dealt with in Table II, where the animals received a similar diet without addition of any paraffin. The third curve in

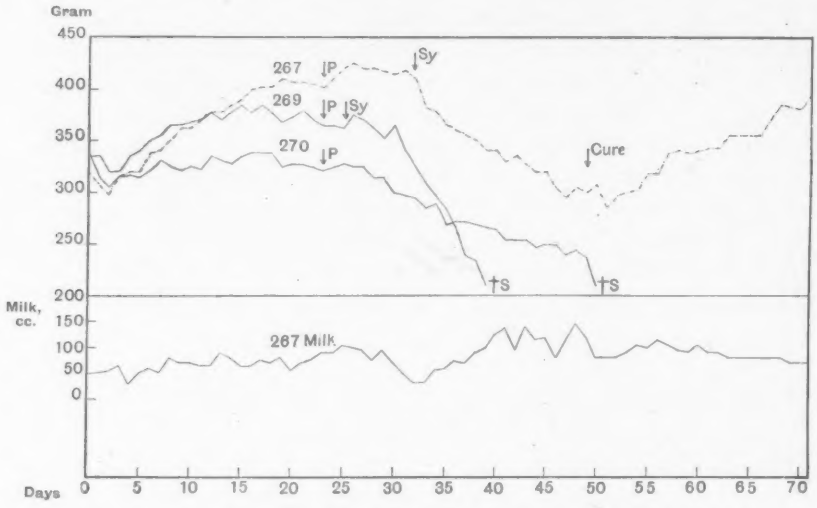


Chart 5. Weight curves of young guinea-pigs upon a diet of oats, bran and autoclaved milk.

↓ P signifies that 1 cc. liquid paraffin was given every second day subsequently.

↓ Sy signifies scurvy symptoms noted.

↓ cure signifies cure with orange juice started, paraffin discontinued.

†S signifies death from scurvy

Lower curve shows daily consumption of autoclaved milk in case of No. 267. It is interesting to note in this case that although the consumption of autoclaved milk increased from the 33rd day, the weight curve continued to fall steadily until after the orange juice was administered. Increased consumption of milk autoclaved at 120° C. for an hour had therefore no beneficial effect in scurvy.

Chart 5 is concerned with guinea-pig No. 267, which developed scurvy on a diet of oats, bran and autoclaved milk, and was first treated with paraffin (as Nos. 269 and 270), but also received no benefit; later this treatment was changed for a daily dose of orange juice and immediate recovery from scurvy began.

Our experiments upon this point, as far as they go, do not, therefore, confirm the conclusion of McCollum and Pitz.

## Application of the Experimental Results to the Question of Infant Feeding.

The foregoing results indicate that anti-scorbutic substances are present in fresh cow's milk, but in small amount. It is therefore clear that when the milk is destined for the use of infants, any method of preparation which tends to diminish this small amount must be regarded with suspicion. Among these processes are included the heating and the drying of milk.

### *Use of Heated Milk.*

It has become the almost universal custom to subject milk to preliminary heating before feeding to infants, in order to prevent transmission of bacterial disease.

Our own experiments with heated milk have only dealt with very severe heating indeed (at about 120° C. for one hour) and the ration taken has as a rule been less than 60 cc., an amount which is insufficient to protect against scurvy even when the milk is fresh. In the few cases, however, in which we have seen much larger amounts consumed, there was no sign that any anti-scorbutic substance was present which could assist in the process either of protection or of cure.

Frölich [1912] found that a diet of oats and milk, heated to 70° C. for 30 minutes or to 98° C. for 10 minutes, produced scurvy in guinea-pigs more frequently than did raw milk, but as he did not measure the quantities consumed, it is not possible to draw quantitative deductions from his work.

Practical experience of infant feeding also affords proof that damage is done when the heating takes place at temperatures lower than boiling point. Neumann [1902] states that in 1901–1902 he encountered 21 cases of infantile scurvy in his private practice, whereas previously he had rarely seen the disease. Of these 21 cases, 20 had for several months received milk from the same dairy, a dairy in which since 1901 it had been the custom to pasteurise the milk at 90–95° C. before delivery. In all cases the milk had been heated a second time after delivery in a Soxhlet or other apparatus, at or near 100° C., for 10–15 minutes. A similar experience is related by Heubner [1903]. This Berlin physician noted only 23 cases of infantile scurvy in Berlin between the years 1894 to 1900 inclusive. In 1901–1902 he met with 59 cases, of which 31 had been fed on milk previously heated in various ways and 11 had received condensed milk or patent food. He, in common with Neumann, attributed the marked increase in infantile scurvy at this period to the introduction of pasteurisation or boiling of milk before feeding to infants.

Perhaps the best instance in the literature is that studied in great detail by Hess and Fish [1914] and Hess [1916] in which 23 cases of mild infantile scurvy were shown to be caused by a diet for several months of cow's milk previously pasteurised at 145° F. (63° C.) for 30 minutes. This outbreak occurred at the Hebrew Infant Asylum, New York, where it had long been the custom to use pasteurised milk for the infants and in addition to give a regular ration of fresh orange juice. After the American Medical Milk Commissions had published the conclusion [1912] that, for purposes of infant feeding, heated milk might be considered as the equivalent of raw milk, the use of orange juice was discontinued in the Infant Asylum. The result was that an outbreak of mild scurvy took place about two to four months later. The babies were not acutely ill, and the symptoms were mild in comparison with the clinical picture of severe infantile scurvy. The patients were fretful, showed marked pallor, loss of appetite and some degree of anaemia; they also ceased to gain weight or to grow in length. In a few cases there were distinct haemorrhages in the mouth and elsewhere and in others a thin blue line of induration could be detected round the margin of the teeth. The infants were all more than six months old, and it would have been reasonable to attribute the illness to teething troubles. The scorbutic nature of the disorder was, however, proved by the fact that all symptoms cleared up speedily after restoring the extra anti-scorbutic to the diet or substituting raw milk for the heated milk.

Miller [1917] has described a series of similar cases from his private practice. The illness was mild, and other diagnoses were suggested, but successful cures following on treatment with anti-scorbutic material proved the trouble to be incipient infantile scurvy.

*Use of Dried Milk.*

Dried milk we believe to be largely, if not entirely, deprived of anti-scurvy vitamine. Whether this happens during the process of drying or in the long keeping which follows drying we are unable to say. We have, so far, discovered no conditions under which the anti-scorbutic vitamine can be preserved intact apart from living tissues for any considerable time at room temperature. It is slowly destroyed by some process at present unknown, and in dried milk there is no exception to the rule.

The history of two guinea-pigs (Nos. 163 and 162) are given in Table II, section (*b*). These animals consumed daily an average amount of dried milk equivalent to 60 cc. and 76 cc. milk respectively and there was no

significant protection from scurvy. The loss of anti-scorbutic principle originally contained in the milk is seen by comparing the results of these two experiments with Experiments 2, 3 and 4 in Table III, where similar amounts of fresh raw milk were consumed.

If infants are nourished on dried milk or on "artificial" milk (prepared from dried skim milk with addition of vegetable fats) nutritive disorders are to be apprehended if the diet is maintained for several months, and if no extra anti-scorbutic is added. Expert medical opinion is divided upon this point, as upon the question of the adequacy of heated milk. Naish [1914], for example, believes that "the risk of scurvy upon such a diet is non-existent," and adds that he is not in the "habit of ordering any orange juice or other anti-scorbutic." Pritchard [1914, p. 304], on the other hand, states that "when dried milks or other preserved foods are employed, it is well to give some fresh fruit juice or other anti-scorbutic."

It must be remembered that scurvy is a disease which develops after a long period of imperfect nutrition, extending usually for several months. The symptoms will vary in severity according to the susceptibility of the individual and to the degree of deficiency in the diet. Every gradation may therefore be expected from almost imperceptible changes in mild cases to the well recognized severe symptoms of acute infantile scurvy. This point merits the consideration of those authorities who deny the need for extra anti-scorbutics in artificial feeding of infants on the ground that infantile scurvy is a very rare disease, in comparison with the great frequency of artificial feeding without extra anti-scorbutic. It is possible, however, that more careful observation may discover that in its milder manifestation, infantile scurvy is not so uncommon an ailment as has been suspected.

It is essential, where infants are concerned, that the very best conditions should prevail and it is the only prudent course, in the present uncertain state of our knowledge, to supply additional anti-scorbutic substance when anything other than fresh cow's milk is given. Of these, orange juice is easily the most suitable, but when the conditions are such as to put it out of the reach of the poor, raw swede juice may be substituted. We have found that when freshly squeezed, it is a valuable anti-scorbutic, though not as potent as orange juice; a larger dose should therefore be given.

Research at this Institute is now in progress to determine the exact relative values against scurvy of these and other foodstuffs, and also if possible to discover other anti-scorbutic materials which may usefully be employed in infant feeding.

SUMMARY.

(1) A set of nutritional experiments with young guinea-pigs is described in which the diet consisted of oats, wheat bran and fresh milk. The special feature of the work is the measurement of the amounts actually consumed.

(2) The basal scurvy-producing diet for control purposes consisted of oats, bran and water, or oats, bran, and 60 cc. milk autoclaved at 120° C. for an hour. With the former diet no growth was produced and it seemed possible that it lacked both the anti-scorbutic factor and the "Fat Soluble A" growth factor of McCollum and his co-workers. With the addition of the autoclaved milk growth took place until the onset of scurvy symptoms; it was concluded therefore that this diet was in every way a sufficient one, save only that it lacked the anti-scorbutic factor.

(3) If the daily consumption of fresh milk was less than 50 cc. the guinea-pig developed scurvy in a period not different from that in the control experiments.

(4) If the daily ration varied from 50–100 cc., a greater or less protection from scurvy was observed, varying proportionately with the amount consumed.

(5) If 100–150 cc. was taken daily, satisfactory growth and development occurred and no symptoms of scurvy were observed. This practically amounts to a complete milk diet.

(6) The above facts accord well with the vitamine deficiency hypothesis of the etiology of guinea-pig scurvy. Milk is evidently a food poor in the anti-scurvy accessory factor and a ration large in comparison with that of other anti-scorbutic materials is necessary to afford satisfactory protection from scurvy.

(7) In our opinion these experiments offer a reasonable explanation of the anomalous results obtained by other observers, when guinea-pigs were fed on diets consisting of grain and fresh milk, and when no measurements were made of the amount of milk actually consumed. Irregularity was noticed in the onset of scurvy symptoms and the observers were led to seek an explanation of the ill-health in causes other than a deficiency in diet. Jackson and Moore [1916] and Jackson and Moody [1916] have arrived at the conclusion that guinea-pig scurvy is an infection of bacterial origin; McCollum and Pitz [1917] think that it is due to chronic constipation, caused by the physical properties of the oats and milk diet.

(8) We have obtained no evidence that chronic constipation is a constant concomitant of guinea-pig scurvy. Instances are given, where certain

modifications in diet, to which no extra laxative effect can be attributed, have cured or prevented guinea-pig scurvy. Experiments are also described in which the administration of a laxative alone has failed to cure or prevent scurvy. The results, on the one hand, afford additional confirmation to the theory that guinea-pig scurvy is due to the deficiency in the diet of a specific accessory substance and on the other hand to the theory that it is analogous in etiology and method of cure with human scurvy.

(9) Application of the results is made to infant feeding with cow's milk and attention is called to the risk of tampering with a substance which even in the fresh condition is but feebly anti-scorbutic. In order to give the child optimum conditions, it is strongly urged that whenever milk is heated in any way, or dried, an additional source of anti-scorbutic vitamine should be provided. Clinical experience and animal experiments point to orange juice as the most suitable anti-scorbutic to employ, but if, owing to war or other conditions, it is unobtainable, raw swede juice in rather larger doses may be substituted.

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# XII. THE EXAMINATION OF THE FAECES OF RABBITS FED ON A DIET OF CABBAGE FOR THE OCCURRENCE OF A PHYTOSTEROL, INCLUDING A NOTE ON THE PHYTOSTEROL IN CABBAGE SEEDS AND THAT IN GRASS FRUITS.

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Previous experiments by Dorée and Gardner [1908] on the excretion of cholesterol in the animal organism, led to the belief that the phytosterols and similar bodies occurring in the plant given as food appear in the faeces in an unchanged form.

The examination of the product when grass was given as food has already been carried out by these authors. The chief body in the faeces under these conditions is chortosterol, an alcohol having the formula $C_{27}H_{54}O$, or $C_{27}H_{56}O$, which does not give the cholesterol colour reactions, and this substance was also found in grass leaves. Phytosterol itself was either absent, or present in the oily matter of the unsaponifiable ether extract in too small amount to separate. When bran was given in the food, the phytosterol of bran was found in the faeces. Further, from results obtained by Fraser and Gardner [1909] it appeared that when phytosterol was given in the food of rabbits, the greater part appeared in the faeces. Further, experiments carried out later [Fraser and Gardner 1910] by the same authors showed that although phytosterol given in the food increased the cholesterol content of the blood, it did not appear in the blood as such, and must have been altered in form or excreted. In continuation of this work, an examination of the faeces of rabbits fed on a diet of cabbage was made by the author, to determine whether a phytosterol was present, and to investigate the change, if any, in this substance in its passage through the digestive organs.

Rabbits in the laboratory were fed on cabbages, some on the outer leaves and others on the inner stem, including the apical bud—40 kilos of leaves, 30 kilos of stalk were used. Their faeces were collected and extracted separately. The account will thus be divided into results obtained with leaves and with stalks.

The faeces were dried, and then ground up as finely as possible. The dried material was subjected to prolonged extraction with ether in a Soxhlet apparatus. The ether extract thus obtained was saponified with a large excess of alcoholic sodium ethylate according to the method of Kossel and Obermüller. In order to insure complete saponification the saponified liquid was always allowed to stand over-night. The precipitate of soap was separated by filtration, and washed with ether to remove all the ether-soluble substances. The filtrate was shaken up with water several times to free it from excess of alkali. Finally the washed ether extract was evaporated to dryness, the residue dried and kept for examination.

*The Leaves.*

On saponification it was observed that very little soap was present. The dried ether extract consisted of solid matter, not obviously crystalline, in a brown oil. This matter proved exceedingly difficult to separate, as the brown oil came out of solution with the solid from ethyl alcohol, acetone, and ethyl acetate. The solution in acetone was therefore boiled with charcoal, and afterwards filtered hot. The charcoal was subsequently extracted several times with boiling acetone to remove the substances as completely as possible. From this solution a pure white substance was obtained in small crystals; in granular lumps from acetone and ethyl acetate. It was finally recrystallised from ether, from which it separates in beautiful rosettes of lustrous crystals.

(1) The first substance to come out of solution in ether melts at 78°–79°. It separates from alcohol as a jelly, in a more granular form from acetone and ethyl acetate; from ether and chloroform in much larger rosettes of crystals. It does not give the cholesterol reactions and does not form an acetate. The similarity in properties made it probable that it might be chortosterol, but as the acetate could not be obtained this could not be proved. The amount of the substance was too small for further investigation, but it was evidently not a phytosterol.

(2) The mother liquors deposited a crystalline solid melting between 60°–75°; this was evidently a mixture and proved very difficult to separate.

Finally a substance of M.P. 63°–64° was isolated which gave a constant melting point after several recrystallisations. It is very light, and separates in a crystalline form from ethyl acetate, acetone, ether and chloroform. From acetone, when the substance is pure, the crystals under the microscope show the form of 4-sided plates. Salkowski's and Liebermann's tests are negative. It has no specific rotation.

From its properties it evidently does not belong to the sterol group, and is probably one of the hydrocarbons or alcohols occurring in plant waxes.

(3) The mother liquors consist of a heavy brown oil, containing a trace of crystalline matter, too small for identification. This gives the cholesterol colour reactions.

*The Stalks.*

The unsaponifiable residue from the faeces of rabbits fed on cabbage stalks was very similar to that from the leaves in appearance. The solid crystallising out was difficult to separate from the brown oil present, and the residue was dissolved in acetone and boiled up with charcoal.

Two substances were isolated.

(1) The less easily soluble substance which came out of solution almost completely in the cold from most solvents melted between 60° and 70°. After repeated crystallisation from ether, a substance melting at 63°–64° was separated in small amount. It had the same crystalline properties as that from the leaves, and the M.P. was not altered by mixing the substances from the stalks and leaves in equal proportions.

(2) The more soluble substance remained behind in the mother liquors. On evaporation to small bulk a distinctly crystalline body separated out from the oily solution in rosettes of long needles. The substance was finally purified from absolute alcohol. The melting point was 102°, and the substance gave the cholesterol colour reactions. Only a very small amount of this substance was present, so that it was difficult to identify it further.

THE TISSUES OF THE CABBAGE.

An examination of the dried leaves and stalks of the cabbage itself showed that the unsaponifiable ether extract was very small in amount. It consisted mainly of the same substances as appeared in the faeces, and although the mother liquors gave the phytosterol colour reactions, no crystalline substance like phytosterol was isolated. It appeared from these results that a typical phytosterol, if present, only occurred in the cabbage plant to a very small

extent, and as a comparative experiment, the seeds were examined to find out if they contained this substance.

*The Seeds.* Two kilos. of cabbage seeds were ground up very finely and extracted with ether. The extract was saponified in the usual way, and the unsaponifiable matter, after drying, weighed 14·9274 g.

It consisted of a mixture of crystalline matter with a brown oil. The residue was recrystallised from alcohol, and finally purified from solution in acetone. The crystals from alcohol were glistening plates, from acetone smaller. They gave the cholesterol colour reactions; the melting point of the pure substance was 142°. A substance with the same melting point was described by Bömer [1899, 1901] and later by Windaus and Welsch [1909] in rape oil. The latter authors were able to show that it was a mixture of two substances: (1) brassicasterol, $C_{28}H_{46}O$, M.P. 148°, and (2) a phytosterol $C_{27}H_{46}O$, M.P. 142°. They obtained the bromide of the acetate, and found that brassicasterol formed a tetrabromide insoluble in glacial acetic acid, whereas the other substance formed a dibromide which remained in solution.

This phytosterol resembles the sitosterol described by Burian [1897] and later by Ritter [1902] in wheat and rye embryos.

In order to determine if the substance isolated from cabbage seeds was the same as that in rape oil the following experiments were carried out.

*Preparation of the acetate.* 0·05 g. of the substance was mixed with 0·1 g. of sodium acetate, and a few cc. of acetic anhydride added. The mixture was boiled under a reflux condenser for ¾ hour and then poured into much water and allowed to stand over-night. The precipitate was then filtered, washed free from acetic acid, and dissolved in alcohol, from which it was twice recrystallised. The crystals melted at 137°. This seemed so similar to the mixtures of substances in rape oil, that it was interesting to observe if a tetrabromide would be formed.

*Preparation of the bromide of the acetate.* A further preparation of the acetate was carried out, and about 0·25 g. was dissolved in 2 cc. of ether, and 3 cc. of a 5 % solution of bromine in glacial acetic acid were added. After some time crystals separated out. To determine if these were identical with those of the tetrabromide of the acetate of brassicasterol, they were purified by recrystallisation from absolute alcohol, but the results were not very conclusive. The tetrabromide of the acetate of brassicasterol described by Windaus and Welsch, melts at 209°. The melting point of the substance just described was lower. It is hoped later to carry out the experiments on the same scale as was done by Windaus and Welsch, who used much larger quantities.

The results obtained from these experiments: that the cabbage seeds contain a relatively large amount of a mixture of phytosterols, whereas the vegetative organs seem to contain a very small amount, made it interesting to compare the investigation of grass described by Dorée and Gardner [1908] with an examination of the fruit.

### Grass Fruits.

515 g. of grass fruits were extracted, giving an unsaponifiable residue weighing 2·05 g. From this residue on crystallisation from acetone, the first substance which separates out in small quantity, resembles chortosterol in its solubility, but it was too small in amount to be purified satisfactorily. It is probably derived from the leaf-structures surrounding the grass fruit, and inseparable from it. From the mother liquors, 0·07 g. of a crystalline substance resembling phytosterol was obtained. This melted at 135°–137° and gave the cholesterol colour reactions.

0·04 g. was converted into the acetate, which after recrystallisation melted at 119°–120°. This remained unaltered on subsequent recrystallisation.

It would seem, from the data available, that this is a mixture of sitosterol and some similar body, but the quantity was too small to attempt separation of the constituents.

### Summary and Conclusions.

(1) The chief constituent of the unsaponifiable ether extract of the faeces of rabbits fed on a diet of cabbage, was not phytosterol, but a mixture of substances of lower melting point, probably hydrocarbons or alcohols such as are known to occur in plant waxes. A typical phytosterol was not isolated either from the faeces or from the vegetative organs of the cabbage plant itself, although a small amount of a substance melting at 102° and giving the cholesterol colour reactions was separated from the faeces of rabbits fed on cabbage stalks. This might be coprosterol, although that substance has never been found in the faeces of the herbivora, but the amount was too small for complete identification.

(2) Cabbage seeds, on the other hand, contain relatively a large amount of crystalline matter, which seemed similar to the mixture of phytosterols present in rape oil. This is interesting as rape and cabbage are different species of the same genus—*Brassica*.

(3) Grass fruits also contain phytosterol, although the chief constituent of the unsaponifiable ether extract of the leaves is chortosterol, which does not give the cholesterol colour reactions.

(4) These results seem to indicate that whereas phytosterol is present in the seeds, in the vegetative organs it is absent or present in relatively small amount.

Such a conclusion, however, would hardly be justified unless some more accurate method of separation were employed, permitting of the separation of phytosterol which may be present in the oily residues, as they give the phytosterol colour reactions.

Further experiments are in progress, and the details will be given in a later communication.

In conclusion the author would like to express her thanks to Professor Waller and to Mr Gardner for the facilities afforded in the Physiological Laboratory.

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# XIII. CONTRIBUTIONS TO OUR KNOWLEDGE OF THE PLANT STEROLS. PART I. THE STEROL CONTENT OF WHEAT (*TRITICUM SATIVUM*).

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In accounts of the chemical analysis of plants, "a phytosterol" is often mentioned among the constituents. Particularly noteworthy in this connection are the researches of Power and his co-workers at the Wellcome Chemical Research Laboratories.

Descriptions of the constituents of plants or parts of plants of pharmaceutical importance have appeared during recent years in the *Journal of the Chemical Society*—and in pharmaceutical journals—and it would seem from these that phytosterol is present in seeds, roots, leaves, shoots, flowers—in latex and in bark.

In addition to this the phytosterols from special sources have been examined very carefully by various authors. The substances so described are isomeric with the animal cholesterol, in most cases having the formula $C_{27}H_{46}O$, but included in the group are certain other compounds possessing slightly different formulae which may be regarded as very nearly related. Chemically, like cholesterol, they react as unsaturated alcohols forming bromine additive compounds, and esters. They are generally optically active, and they give the cholesterol colour reactions—though sometimes in a modified form. The two chief tests of this kind are:

(1) *The Salkowski Test*—in which a few mg. of the substance are dissolved in about 2 cc. of chloroform and shaken with an equal bulk of strong sulphuric acid. The chloroform develops a blood-red or cherry-red colour which it retains for several days. On pouring this solution into a basin, the colour changes to blue, green, and then yellow, owing to absorption of water. On addition of sulphuric acid, the original colour is restored. The sulphuric acid layer is distinctly fluorescent.

(2) *The Burchardt-Liebermann Test.* A few mg. of the substance are dissolved in 2 cc. of chloroform, 20 drops of acetic anhydride added, and one drop of concentrated sulphuric acid. A violet pink colour appears. This test will detect very small traces of sterol, but it is also given by resins.

Phytosterols usually have a characteristic crystalline form. From hot alcoholic solution, bundles of glistening crystals appear on cooling, and, when slowly crystallised, these are found to be well-defined hexagonal plates.

Whereas cholesterol itself is found to be a constant constituent of animal protoplasm, in the vegetable kingdom it seems that a number of isomers exist, differing from one another to a slight extent in their properties, notably in their melting points and the melting points of their esters. These substances form mixtures which crystallise as if only a single substance were present. Mixtures of the plant sterols with cholesterol (which has not so far been found in plants) behave in the same way. Many of the substances described melt between 130° and 138°—but compounds with higher melting points have been described.

The differences in these substances are not much greater, however, than those observed in the case of the five dihydrocholesterols which have been described and which can easily be changed one into the other, as has been shown by Dorée and Gardner [1908, 1] in the case of coprosterol and $\psi$-coprosterol and in the case of the rest by Windaus and his co-workers [Windaus and Uibrig, 1914, 1915].

According to the researches of Windaus [1916] these bodies possess in a high degree the capacity of crystallising together so as to simulate a single compound. The various isomers in Windaus' opinion differ only in the stereo-position of H and OH groups with regard to an asymmetric carbon atom.

These varying plant sterols, as already indicated, are very widely distributed. The best known perhaps are those occurring in:

(1) *Seeds.* Hesse [1878] gave the name "phytosterol" to a substance present in Calabar beans, to which he assigned the formula $C_{26}H_{44}O + H_2O$. It melted at 132–133° and formed an acetate, M.P. 120°.

A similar substance had been described by earlier writers, Beneke [1862] in seed peas, Ritthausen [1863] in wheat gluten, Lindenmeyer [1863] in peas, but these authors considered it to be cholesterol. After that, a number of papers appeared describing phytosterols melting between 132° and 137°.

The formulae assigned often differ slightly from $C_{27}H_{46}O$, but the method of combustion is not entirely conclusive unless many determinations of the substance and as many esters as possible are made, since the percentage

carbon and hydrogen content would differ but little in compounds of such high molecular weight.

The phytosterols described are isolated in very small quantities, and one needs a relatively large quantity for satisfactory investigation.

Jacobson [1888] described the phytosterol of peas and beans, and in 1897 a great advance was made by Burian [1897] who isolated from wheat and rye embryos a substance which he named sitosterol, and considered to be isomeric with cholesterol. This melts at 137·5°. It is soluble in most organic solvents, crystallises from 90 % alcohol with one molecule of water of crystallisation and in ether has $[\alpha]_D = -26°{\cdot}71$.

He prepared many of the derivatives of sitosterol and characterised the substance fairly completely. The acetate melted at 127° and the benzoate at 145–145·5°. Cholesterol benzoate shows a fine play of colour on cooling, and sitosteryl benzoate does this in a less pronounced fashion.

Ritter [1902] confirmed this work, and added to our knowledge of the phytosterol from wheat germ. After sitosterol had been thus characterised, it seemed probable that the phytosterols described as melting between 132° and 137° consisted of this substance with varying amounts of some similar compound as impurity.

Owing to the resemblance in their properties the separation of these mixtures presented unusual difficulty.

Windaus and Hauth [1907], however, were able to show that the original phytosterol of Hesse, from Calabar beans, was really a mixture of sitosterol, identical with that in wheat germ, and another alcohol, stigmasterol, M.P. 170°, having the formula $C_{30}H_{48}O$. This latter compound has two double linkages in the molecule and the acetate forms a tetrabromide. This tetrabromide settles out from glacial acetic acid on standing while the sitosteryl acetate dibromide remains in solution.

(2) *In Oils.* As already mentioned, phytosterol has been described as a constituent of many plant oils, notably by Bömer [1899] in cotton-seed oil, ground-nut oil, sesamé oil, colza oil, poppy oil, hemp-seed oil, linseed oil and castor oil. He finds that the M.P. differs slightly according to the source, but the elementary composition is the same. Sani [1903] finds a phytosterol in olive oil, and rape oil was examined first by Bömer [1899, 1901] and later by Windaus and Welsch [1909]. The latter authors showed it to be a mixture, and, by means of the bromide of the acetate, isolated two separate sterols—one melting at 148° which they named brassicasterol. This had the formula $C_{28}H_{46}O$, and like stigmasterol formed a tetrabromide of the acetate. The

other had a lower melting point, 142°, and the authors found that this was a phytosterol with the formula $C_{27}H_{46}O$.

Cocoa butter was found to contain a mixture of two phytosterols by Matthes and Rohdich [1908, 1, 2] but Heiduschka and Gloth [1908] consider that cotton seed oil contains a single substance as no tetrabromide is formed.

The capacity of mixtures of cholesterol and the plant sterols to crystallise as a single substance, is made use of in the detection of adulteration of animal oils by those of plant origin. The characteristic four-sided tablet of cholesterol is always altered by an admixture of phytosterol.

(3) *In Roots and Rhizomes.* Perhaps the earliest mention of the substance occurs in the analysis of carrots by Husemann [1861] under the name of hydrocarotol. This was later described by Euler and Nordensen [1908] under the name of daucasterol. Tutin and Clewer [1912] describe a phytosterol verosterol, from rhubarb rhizome, and Power and Salway [1914, 1] from sarsaparilla root. Rumpler [1903] gives the name betasterin to a phytosterol of M.P. 117° from beetroot.

(4) *In Stem, Leaves and Flowers.* Phytosterols have been found in the flowers of plants, in those of *Anthemis nobilis* for example, as anthesterol, by Klobb [1902] and also by Power and Browning [1914], in the flowering branches of *Clematis vitalba* by Tutin and Clewer [1914] and in the leaves and stems of *Daviesia latifolia* by Power and Salway [1914, 2]. Certain substances analogous to phytosterol have been found in olive leaves by Power and Tutin [1908]: oleasterol, $C_{20}H_{33}OH$, M.P. 174°, olestranol, $C_{25}H_{42}O_2$, M.P. 217–218° and homo-olestranol, M.P. 210°.

(5) *In Latex.* Cohen [1908] has obtained mixtures of phytosterols from South African rubber, and various other bodies from latex have been described.

In addition to the isolation of phytosterol itself as a constituent of plants, Power and his co-workers made the very important discovery that it was present as a glucoside. In their analyses of plants there often occurred high-melting compounds, which seemed to be alcoholic in nature, with more than one hydroxyl in the molecule. These gave the cholesterol colour reactions, but the M.P. and the formulae served to differentiate them from true phytosterols.

As an example, ipuranol, $C_{23}H_{38}O_2(OH)_2$, M.P. 285–290°, occurred in the stems of *Ipomoea purpurea*, in olive bark and in nutmeg. In taraxacum root, cluytianol was found, bryonol in bryony root, and similar substances in various plant organs. A list is given in the paper by Power and Salway [1913] which describes their identification as phytosterol glucosides. They were

unchanged by the usual methods of hydrolysis, but when heated with aqueous hydrochloric acid in amyl alcohol solution, dextrose and a phytosterol were formed. Different phytosterols were obtained from different glucosides and it is probable that different sugars may form part of the molecule.

These phytosterol glucosides are named phytosterolins. They may occur in the plant as well as phytosterol, but in the leaves of *Prunus serotina*, according to Power and Moore [1910], phytosterol is not present except in combination as a glucoside.

The foregoing evidence of the occurrence of a phytosterol in so many different plants and different organs of plants, suggests that in the higher plants, at least, it is a constant constituent. In the lower plants comparatively few phytosterols have been isolated, and these are described chiefly in the fungi.

The best-known representative is the ergosterol of Tanret [1889]. This was first isolated from ergot, melted at 154°, and gave an acetate melting at 159–175°. Later it was further examined by the same author [Tanret, 1908] and shown to be a mixture of two substances: (1) ergosterol, $C_{27}H_{42}O$, melting at 165°, forming an acetate melting at 185°, and possessing an unusually high specific rotation; and (2) fongisterol, the lower homologue, $C_{25}H_{40}O$, M.P. 144°; this is more soluble in the different solvents, has a much lower specific rotation, and gives an acetate melting at 158·5°. These substances are further distinguished by a colour test. Ergosterol gives no colour with strong sulphuric acid at the end of one minute, while fongisterol becomes coloured ruby-red after some seconds, changing to red-violet later.

Gerard [1892, 1895] found an ergosterol present in *Penicillium glaucum*, and *Aethalium septicum*, in *Mucor mucedo*, and in *Lobaria pulmonacea*, and he divides the plant sterols into two groups: (1) the phytosterols, (2) the ergosterols. The first group occur in the higher plants, and usually have lower melting points. The second group occurs in the lower plants, and have higher melting points. The two groups are distinguished by their colour reactions. Zellner [1905, 1908, 1, 2, 1911] describes similar bodies in fat of ergot, in *Amanita muscaria* (the fly agaric), in *Trametes suaveolens* and in *Polyporus ignarius*.

A phytosterol is described in all seeds examined, and in vegetable oils derived from seeds and fruits, but no experiments have hitherto been carried out to trace the fate of this phytosterol in the subsequent development of the plant. It has been described as occurring in all the different organs, but it seemed important to ascertain whether its occurrence was limited

to any special organ, and further if it were possible from such occurrence to form any conclusion as to its probable function.

The results of the examination of the faeces of rabbits fed on a diet of cabbage, of cabbage leaves and stems, and of cabbage seeds [Ellis, 1918], led to the conclusion that while phytosterol was present in relatively large amounts in the seed, the quantity in the vegetative part was small. It seemed necessary to test this conclusion by some more systematic method of experiment, and if possible by the employment of accurate quantitative means. The brown oils giving the cholesterol colour reactions may contain appreciable amounts of a crystalline phytosterol, although it is very difficult to isolate it by the ordinary methods of solution and crystallisation.

For this purpose the wheat plant was chosen partly because the sitosterol from the germ has been so fully characterised by Burian [1897] and later by Ritter [1902], and partly because this sitosterol forms a compound with digitonin to be referred to later. Its economic importance also gives experiments on wheat a value from the utilitarian point of view.

The following experiments were carried out:

(1) The amount of phytosterol present in the whole grain was estimated, and this substance was characterised. Bran and wheat germ meal were also examined, the two latter representing the seed-coat and the germ respectively as nearly as it is possible to obtain them on a large scale.

(2) A known weight of grain was planted and the resulting wheat allowed to grow under normal conditions until well developed. The plants were then carefully collected and the phytosterol content examined.

(3) In addition to the normally developed plants, experiments were carried out with etiolated plants.

Leaves and roots were examined separately in certain of the experiments.

In every case the unsaponifiable ether extract, which would contain any sterol present, was obtained in the following way. The plant material was dried, and then ground up very finely in order to render all the tissues permeable. The dried material was then subjected to prolonged extraction with ether in a Soxhlet apparatus. The ether extract so obtained was saponified with a large excess of alcoholic sodium ethylate, according to the method of Kossel and Obermüller. In order to insure complete saponification the saponified liquid was always allowed to stand over-night. The precipitate of soap was separated by filtration, and washed with ether to remove all the ether-soluble substances. The filtrate was then shaken up with water several

times to free it from excess of alkali. Finally the washed ether extract was evaporated to dryness, the residue dried and examined.

These experiments and their results are given in detail below.

*The Grain.*

3·05 kilos. of grain were ground up very finely and extracted with ether. The ethereal extract was saponified, washed, and the ether evaporated to dryness. The residue consisted of crystalline matter mixed with oil. After drying it weighed 1·528 g. From this about 0·95 g. of crystalline substance was obtained, giving a yield of approximately 0·031 %.

The pure crystals resemble phytosterol and give a melting point of 137·5°. They give the cholesterol colour reactions. This appears to be the same body as that described by Burian and Ritter in the embryo. To identify it further the acetate was prepared in the way previously described. After recrystallisation from acetone, the dried crystals melted at 127°, showing that the chief phytosterol present in wheat grain is that of the embryo—sitosterol.

*The Bran.*

1·25 kilos. of bran were extracted, saponified, and the resulting ether extract evaporated to dryness. The residue weighed 1·267 g. It was repeatedly recrystallised from 95 % alcohol, and finally a substance was obtained melting at 142° which gave the cholesterol colour reactions and resembled phytosterol in crystalline form. This was converted into the acetate by heating with acetic anhydride and sodium acetate, and the resulting compound was recrystallised. It melted at 137°. The benzoate was prepared by dissolving the substance in dry pyridine and adding benzoyl chloride until a faint yellow colour appeared. After standing over-night this was poured into much water and filtered. The precipitate was dissolved in hot alcohol and recrystallised from that solvent. Under the microscope the crystals appeared as hexagonal plates. They melt at 133–134°. The esters on saponification yield the original substance, melting at 142°.

This phytosterol differs from sitosterol in the melting point of its esters, but for its further characterisation much more material would be necessary than was available.

*The Wheat Germ Meal.*

1·8 kilos. of meal were extracted. The unsaponifiable residue consisted almost entirely of crystalline matter and weighed 10·522 g. This gives a yield of about 0·5 %.

### The Normal Wheat Plants.

500 g. of autumn wheat were planted in November, and allowed to grow until May of the following year. These plants were grown at the Chelsea Physic Garden, and the author takes this opportunity of thanking the Curator, Mr Hales, for his very kind help in superintending the plants. The plants were collected as carefully as possible and shoots and roots dried separately. The total dry weight of plants was 2250 g. When dry they were well ground up and extracted with ether.

Shoots and roots will now be described separately.

*The Shoots.* The dry shoots weighed 1950 g. This material was well ground up and extracted with ether. The weight of ether extract obtained was 125·69 g. It was very dark green, greasy looking, and had a curious strong odour. 48 g. of this was taken for saponification. The unsaponifiable residue weighed 5·489 g. The residue was dark orange colour, oily, with characteristic odour. It consisted of a solid mixed with a brown oil, but was not obviously crystalline. This residue was dissolved in alcohol, from which it separates in a jelly-like form, very difficult to filter. When ethyl acetate is employed as a solvent, the substance comes out in more granular form.

It was recrystallised from this solvent, but it was only obtained pure after repeated recrystallisation. The first crop of substance melted at 80°, after recrystallising at 83°. It was so difficult to separate further quantities of this solid from the oil, that a second portion, 37·73 g., of the ether extract was saponified. The unsaponifiable extract, which weighed 4·4772 g., was dissolved in ethyl acetate and boiled up with about an equal quantity of charcoal, the charcoal being subsequently repeatedly extracted with boiling ethyl acetate.

*Alcohol* ($C_{20}H_{42}O$). The ethyl acetate solution yielded a pure white substance after several recrystallisations. It separates from ethyl acetate and acetone in granular lumps of small crystals. The pure substance melts at 83°. The elementary composition corresponds most nearly to $C_{20}H_{42}O$.

0·1024 g.; 0·3016 g. $CO_2$; 0·13365 g. $H_2O$.

| Calculated for $C_{20}H_{42}O$ | Found |
|---|---|
| C = 80·54 | C = 80·32 |
| H = 14·09 | H = 14·50. |

It was converted into the acetate in the usual manner, and the resulting substance purified by recrystallisation from acetone. The acetate was very difficult to filter on account of its soft consistency. It melted at 65°. The

substance did not give the cholesterol colour reactions, and was not a phytosterol. Etard [1892] describes an alcohol, medicagol, from lucerne leaves to which he assigns the formula $C_{20}H_{42}O$. He says it forms a benzoate, but does not give figures. An interesting point about his paper is that he mentions experiments, the results of which showed that when a horse was fed with lucerne leaves, the alcohol was unaltered by passage through the digestive tract of the animal, and appeared in the faeces. This agrees with the conclusions arrived at by Dorée and Gardner [1908, 2].

Haller [1907] describes an alcohol occurring in the wax from *Raphia ruffia*, M.P. 80°, to which the formula $C_{20}H_{42}O$ is assigned, but no figures are given. A mixed melting point with the medicagol of Etard (from whom he obtained a specimen) shows that they are not the same. The acetate described by Haller seems to be the same as that obtained by the author, M.P. 65°, who concludes that the alcohol occurring in wheat leaves is the same as that in *Raphia ruffia*. It is not identical with arachyl alcohol, which melts at 71°. As it was not a phytosterol, the investigation of this substance was not carried further. The mother liquors deposited crystalline matter resembling phytosterol, but the small amount present was difficult to separate from the brown oil accompanying it in order to weigh it directly.

A method of estimation by means of the compound with digitonin was carried out. Windaus [1909] discovered that cholesterol, but not cholesterol esters, combines quantitatively with digitonin to form a highly insoluble compound, digitonin cholesteride, according to the following equation:

$$C_{55}H_{94}O_{28} + C_{27}H_{46}O = C_{82}H_{140}O_{29}.$$

This compound is insoluble in water, acetone, ethyl acetate, ether and benzene.

The residue containing the sterol is dissolved by boiling in 95 % alcohol, and to this solution is added excess of digitonin in 95 % alcohol. The mixture is allowed to stand for some time, then evaporated to dryness. The precipitate is washed by decantation with ether into a previously weighed Gooch crucible until the ethereal washings give no residue on evaporation. After that it is boiled up with water, transferred to the crucible and washed with hot water until the washings no longer froth on being shaken. The compound is somewhat hygroscopic, and the crucible and contents are therefore dried for some time at 110° and weighed in a stoppered vessel, the drying being continued until the weight is constant. This method has been worked out by Gardner and the author [Fraser and Gardner, 1910] in order to estimate small amounts of cholesterol in animal tissues. The amount of digitonin available was not

sufficient to allow of the method in its application to the plant sterols being worked out completely.

It is hoped that the author may carry out that investigation at some future period.

For the purpose of this research, however, use was made of the fact that Windaus found that sitosterol and other well-known isomers of cholesterol formed analogous compounds with digitonin, with similar solubilities. A preliminary investigation with the pure substance, sitosterol, was carried out, and it was found that 0·001 g. sitosterol could be accurately measured by this means. In the case of wheat stalks the alcohol $C_{20}H_{42}O$ was removed by filtration, and washed with acetone until it no longer gave the cholesterol colour reactions. The filtrate and washings were then evaporated to dryness, dried and weighed. The weight was 1·752 g. As the amount of digitonin was limited and there was not enough to precipitate the whole of this residue and those of the other material to be presently described, the following procedure was adopted. The residue was dissolved in 20 cc. of 95 % alcohol, the solution well mixed and 2 cc. removed for the estimation, this forming a tenth part of the whole residue. The weight of compound obtained was 0·1087 g. equivalent to 0·2639 g. phytosterol from the residue from 37·73 g. This gives a total weight of 0·8802 g. phytosterol from 125·69 g., the weight of the ether extract of the shoots. On a dry weight of 1950 g. the percentage content of phytosterol is thus 0·045.

*The Roots.* The dried wheat roots from the normal plants weighed 300 g. The ether extract weighed 1·454 g. It consisted of a small amount of a solid apparently the same as that in the shoots and a brown oil. There was some appearance of crystalline matter. Exactly the same procedure was carried out as for the estimation of phytosterol in the shoots. The residue gave 0·113 g. of sterol, or a percentage of 0·037 on the dry weight of the roots.

### Etiolated Wheat Plants (1).

500 g. grain were planted at the Chelsea Physic Garden in November. They were covered with canvas bags to exclude the light. A few of the plants showed green in spite of this precaution, but in March signs of withering began. The plants were then carefully collected and dried, shoots and roots being separated.

The total dry weight of the plants was 203·5 g.

*The Shoots.* 203·5 g. dried plants gave 135·2 g. dry weight of shoots. This was extracted with ether, and saponified as before. The unsaponifiable

ether extract weighed 2·175 g. It consisted chiefly of the alcohol $C_{20}H_{42}O$ present in the normal shoots, melting at 83°, recrystallised from acetone.

From solution in acetone the mother liquors deposited a distinctly crystalline residue. The procedure adopted for the normal shoots was carried out. The amount of phytosterol present in the residue was 0·0674 g. a percentage of 0·0487 on the dry weight of shoots.

*The Roots.* The dry weight of the roots was 68·5 g. The unsaponifiable ether extract weighed 0·5702 g. This gave 0·0855 g. phytosterol, a percentage of 0·1252 on the dry weight of roots.

### Etiolated Wheat Plants (2).

383 g. wheat grain were grown on filter paper in an incubator in the laboratory. The plants, when grown, were completely etiolated. The dried material weighed 168 g. The unsaponifiable extract from this weighed 1·404 g. The amount of phytosterol present was 0·1175 g. or 0·07 % of the dry weight of plants.

These results are put together in the following table:

*Table showing Sterol Content of Wheat grown under varying conditions.*

| Experiment | | Weight of grain planted in g. | Dry weight of material in g. | Total dry weight of plants in g. | Amount of phytosterol in g. | Total amount of phytosterol in plants in g. | Percentage of phytosterol in roots and shoots | Total percentage of phytosterol | Amount in grain planted (0·031 %) in g. | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal Wheat Plants | shoots | 500 | 1950 | 2250 | 0·8802 | 0·9932 | 0·045 | 0·04 | 0·155 | Increased during growth |
| | roots | | 300 | | 0·1130 | | 0·037 | | | |
| Etiolated Wheat Plants | shoots | 500 | 135·2 | 203·5 | 0·0674 | 0·1529 | 0·0487 | 0·075 | 0·155 | About the same as in seed |
| | roots | | 68·3 | | 0·0855 | | 0·1252 | | | |
| Etiolated Wheat Plants | | 383 | | 168 | | 0·1175 | | 0·070 | 0·1187 | About the same as in seed |

### Summary and Discussion of Results.

(1) In order to obtain more information as to the relative amounts of phytosterol occurring in the different organs of the plant, those of the wheat plant were carefully examined. Schultze and Barbieri [1882] give some figures for the phytosterol content of the seeds and seedlings of lupin plants, but their methods are open to criticism, and they are not certain of their results. The chief phytosterol present in the grain was found to be the same

as that in the embryo—sitosterol—and as the solid part of the unsaponifiable ether extract of the grain consisted entirely of this substance, it could be separated and weighed directly. A phytosterol was present in bran, but it differed from sitosterol in its melting point, and in the melting points of its esters.

(2) In the case of the vegetative organs, other substances were present in the unsaponifiable ether extract—an alcohol $C_{20}H_{42}O$, which did not give the phytosterol colour reactions and was not precipitated by digitonin, and an oil, and it proved difficult to separate the phytosterol in order to weigh it directly. The researches of Windaus and his co-workers have shown that all the phytosterols hitherto isolated from the higher plants, which have been fully characterised, combine with digitonin to form a compound insoluble in ether, acetone, and ethyl acetate. A method of estimation based on this fact was devised, and it was found to be very accurate when applied to pure sitosterol in preliminary experiments.

(3) An estimation of the amount of phytosterol present in the normal wheat plant, grown from a known amount of grain, shows that the quantity in the adult plant is greater than in the grain, whereas in the etiolated plant the amount is very much the same as that in the grain, allowing for experimental errors. The percentage of phytosterol present in the normal plant is slightly greater than the percentage present in the grain, and in the etiolated plant it is higher still, no doubt correlated with the loss in dry weight, due to starvation.

(4) In the embryo the percentage of phytosterol present is much higher than in the plant. This points to an essential function in germination and growth. As the amount present in the grain is not appreciably altered in the starved plant, there is distinct evidence that this function is *not* a nutritive one. This seems an important point and is rather in agreement with the nature of phytosterol and the fact that it is not easily altered chemically. Its occurrence in the seed, in the seed coat, in the embryo, the root and the shoot, in the case examined, suggest a function common to all of them. While no definite conclusions can be drawn until a larger number of investigations have been made, the theory that the sterols may form an essential part of cell membranes suggests itself, but the presence of phytosterol in latex, unless it occurs there as the result of cell-destruction, and in the form of glucosides, is hardly accounted for by this theory. Many experiments will still have to be carried out before we can form any very definite conclusion as to the function of phytosterol in the plant.

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# XIV. CONTRIBUTIONS TO OUR KNOWLEDGE OF THE PLANT STEROLS. PART II. THE OCCURRENCE OF PHYTOSTEROL IN SOME OF THE LOWER PLANTS.

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In the first paper of this series [1918], an account of the phytosterols occurring in the lower plants is given. These are represented chiefly by two substances occurring in the fungi: (1) ergosterol, first isolated by Tanret [1889], and (2) fongisterol, which have been obtained by Tanret [1908] and other workers. Gerard [1892, 1895] puts forward the theory that the sterols may be divided into two groups: (1) the Phytosterols occurring in the higher plants, and (2) the Ergosterols found only in the lower plants. To obtain more knowledge on this matter, several species, chosen from different groups belonging to the lower plants, were examined for the occurrence of a phytosterol.

The following were dealt with:

(1) Musci, *Sphagnum.*
(2) Algae, *Laminaria.*
(3) Fungi, (i) *Polyporus nigricans.*
(ii) *Polyporus betulinus.*
(iii) *Agaricus rubescens.*
(iv) *Lactarius subdulcis.*

*Method of Experiment.*

The plant material is dried, and then ground up very finely in order to render all the tissues permeable.

In the case of the juicy fungi, the material is ground up in a mortar with coarse sand, adding plaster of Paris until the mixture hardens. When it is dry, the mixture is coarsely powdered.

The dried material in either case is then subjected to prolonged extraction with ether in a Soxhlet apparatus. The ether extract so obtained is saponified

with a large excess of alcoholic sodium ethylate, according to the method of Kossel and Obermüller. The saponified liquid is always allowed to stand overnight to insure complete saponification. The precipitate of soap is separated by filtration, and washed with ether to remove all the ether-soluble substances. The filtrate is shaken up with water several times to free it from the excess of alkali. Finally the washed ether extract is evaporated to dryness, the residue dried, and examined. Further details will be given for each experiment.

1. *Musci. Sphagnum.* Plants were gathered in the autumn and dried. The dry weight was 622 g.

After saponifying and drying, the ethereal extract showed no sign of crystalline matter, consisting mainly of a brown oil. This oil, however, gave the cholesterol colour reactions.

2. *Algae. Laminaria.* The seaweed was dried in air and "stalks" and fronds were extracted separately. The dry weight of the fronds was 7·132 kilos., and of the "stalks" 1·516 kilos. They were extracted with ether, and the ether extract saponified as usual. Incidentally it was observed that while the ethereal extract from the "stalks" was brown, that from the fronds was green and fluorescent. This points to further modifications in the direction of "stem" and leaf function than the mere external appearance. The pseudophloem found in the stalk gives histological support to such a suggestion.

*The Fronds.* The unsaponifiable extract from the fronds weighed 18 g. It consists mostly of a dark brown oil with a curious penetrating odour. A very small amount of crystalline matter was separated by recrystallisation from alcohol in sheaves of needle crystals, melting at 105°–110°. These gradually turned brown in colour as they were heated from 50°. On further purification with alcohol they melted at 125°.

On standing for some time these crystals seemed to change, lost their form and broke down to an amorphous powder, but the amount was so small that it was impossible to investigate this phenomenon further.

*The Stalks.* The unsaponifiable extract from the stalks weighed 3·6 g. It consisted almost entirely of a brown oil, only a trace of crystalline matter being separated. This resembled the needle crystals from the fronds.

The bulk of the unsaponifiable matter in both cases consisted of a brown oil which gave the cholesterol colour reactions. This was kept, and from time to time attempts were made to obtain crystalline matter from it from solution in various solvents. It did not apparently change on standing, however, or deposit crystalline matter, and it was thought possible that this oil itself

might be a form of sterol. The oil was therefore distilled in steam, when it came over as a pale yellow liquid which darkened very quickly on exposure. This oil gave the cholesterol colour reactions distinctly.

This evidently opens out fresh fields of investigation. Windaus was able to separate the various isomeric forms of dihydrocholesterol by means of digitonin, as they do not all form a compound with this substance, and it is thought that this fact gives a suggestion in regard to the separation of these plant sterols.

The oils from this and other material are being kept. Their examination and identiffcation obviously entail somewhat lengthy investigation, but it may lead to important discoveries in regard to the occurrence of sterols.

3. *Fungi.* (i) *Polyporus nigricans.* The *Polyporus* was gathered in the autumn. The total dry weight of fungi was 4·1 kilos., which gave an unsaponifiable ether extract of about 40 g.

The fungi were extracted in two portions. The first portion of 1050 g. gave an unsaponifiable ether extract weighing 9·6 g. This residue consisted of a dark brown oil, mixed with crystalline matter. The whole was dissolved in 95 % alcohol. (1) The first crop of pure white crystals melted at 248°–249°; on further recrystallisation the M.P. was 250° and this remained constant. The substance was obtained from alcohol in beautiful sheaves of needle crystals. They did not give the cholesterol colour reactions, and were not investigated further at the time. The mother liquors yield a little more of this substance, not quite pure.

(2) The bulk of the more soluble substance, however, melted from 158°–167° and finally a substance giving a constant melting point of 163° was obtained. This was converted into the acetate which melted at 158°. This proved to be a mixture, however, and the author succeeded in separating the constituents by their different solubility in acetone. The less soluble substance melted at 185° after several recrystallisations, and was identified as ergosteryl acetate; this yielded ergosterol, M.P. 165°, on saponification. The acetate of fongisterol, M.P. 158·5°, remained in solution, and was obtained from the mother liquors. Fongisterol itself melts at 144°.

These substances were further identified by a colour test. Ergosterol and strong sulphuric acid give no colour at the end of a minute, whereas fongisterol colours red, then red-violet at the end of a few seconds, so that the crystalline sterols in this *Polyporus* were identified as a mixture of ergosterol and fongisterol—described by Tanret in other fungi.

A quantity of heavy brown oil was left and for some time no more crystal-

line matter could be extracted. On standing, however, for a long period, it was found that the residues yielded more crystalline matter, similar to the mixture described above, when dissolved in glacial acetic acid. Alcohol, acetone and ethyl acetate were not found to produce the same result.

The residue consists of a heavy, almost clear brown oil, which gives the cholesterol colour reactions, and is being kept for future investigation.

In the case of the second portion of the fungi, the unsaponifiable residue was a sticky, gummy, yellow substance. This dissolved in ether, alcohol, acetone, ethyl acetate, and acetic acid, but came out of solution in the same form and no crystalline matter was obtained.

For some time, all efforts to crystallise this substance failed, but, after it had stood for a considerable period, a further attempt was successful, and the solution in acetone deposited crystalline matter identical with that already described, a less soluble substance melting at 250° and the more soluble mixture of ergosterol and fongisterol.

This seems to give an indication that these bodies may change on standing, from one form to another, or that some process of oxidation occurs on standing.

(ii) *Polyporus betulinus*. The fungi were gathered in the autumn, dried, ground up, and extracted with ether. The weight of fungi was 950 g. which gave an unsaponifiable ether extract weighing 5·48 g.

The extract contains a very little crystalline matter, which melts at 157° and gives a colour test with strong sulphuric acid resembling that of fongisterol, blood-red, then red-violet at the end of a few seconds. It is probably a mixture like that occurring in the other fungi, but the amount was too small for separation of the constituents.

The main bulk of the unsaponifiable ether extract consists of a heavy brown oil, which gives the cholesterol colour reactions. It did not yield any further crystalline matter after standing a considerable time.

(iii) *Agaricus rubescens* (5·5 kilos.) and (iv) *Lactarius subdulcis* (3·5 kilos.) were also examined. The material, consisting of young plants, was ground up with sand and plaster of Paris and allowed to dry. The ethereal extract was saponified and the unsaponifiable ether extract obtained in the usual way. This showed no sign of crystalline matter in the case of either fungus. The residues consisted of brown oils which, however, gave the cholesterol colour reactions.

## Summary of Results.

(1) From the fungus, *Polyporus nigricans*, and probably also from *Polyporus betulinus*, a mixture of two sterols previously known to occur in the fungi—ergosterol and fongisterol—was isolated.

(2) There is a certain amount of evidence that in the lower plants at least, the sterols may occur in a non-crystalline form, as in the alga—*Laminaria*—an oil was obtained by distillation in steam, which gave the cholesterol colour reactions, and the unsaponifiable ether extracts from *Sphagnum* (Musci), *Agaricus rubescens* and *Lactarius subdulcis* (Fungi) consist of brown oils, giving these tests. Also on standing the substances seem to change from one form to another. In *Polyporus nigricans* the unsaponifiable ether extract was at first gummy and sticky and no crystalline matter could be isolated. After standing for some time, however, crystals were obtained from solution in acetone.

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